Dependence of Mononucleosome Deoxyribonucleic Acid Conformation on the Deoxyribonucleic Acid Length and H1/H5 Content. Circular Dichroism and Thermal Denaturation Studies†

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ABSTRACT: Four mononucleosome preparations were isolated from micrococcal nuclease digests of chicken erythrocyte nuclei which differed in average deoxyribonucleic acid (DNA) length and in H1 and H5 content. The circular dichroism properties of the unperturbed mononucleosome preparations and the corresponding H1- and H5-depleted species demonstrate that the nucleoprotein spectra above 250 nm are all altered relative to protein-free DNA by the addition of a single negative band at 275 nm, similar to the band observed for Ψ-DNA. The quantitative analysis of the Ψ-type band intensity for any of the higher molecular weight unperturbed samples relative to core particle mononucleosomes yielded a constant number of DNA base pairs (~140) contributing to this new band. Upon removal of H1 and H5 from the mononucleosome preparations which have sufficiently long linker DNA, the Ψ-type band intensity indicates an ~30 base pair reduction in the number of core DNA base pairs contributing to the altered circular dichroism properties. The Ψ-type band is proposed to be due to the compact DNA tertiary structure, i.e., the manner in which the DNA is wound around the histone core allowing interactions between adjacent turns of the superhelix. This interpretation implies that ~30 base pairs of core DNA are removed from the unique core tertiary structure when the linker DNA is not bound by H1 or H5. The circular dichroism analysis correlates well with the thermal denaturation properties of mononucleosomes. Removal of H1 and H5 causes an overall reduction in the thermal stability of both core and linker DNA. The degree of destabilization is greatest when the average DNA length is maximum. Some core DNA is lost from the highest temperature melting bands when histone-free DNA is present. These results indicate two regions of different conformational and thermodynamic stability in core DNA. The length of attached linker DNA and its histone content influence the two regions of the core to differing extents.

The repeating subunit of chromatin, termed the nucleosome, consists of a core particle with ~140 base pairs of DNA wrapped around a histone octamer and a linker DNA segment to which the very lysine-rich histone H1 (and H5 in avian erythrocytes) is bound [see reviews by Kornberg (1977) and Felsenfeld (1978)]. The binding site of H1 is situated close to the core histone octamer (Bonner, 1978; Glotov et al., 1978). The linker DNA tertiary structure is sensitive to factors such as the mode of sample preparation, the solvent conditions, and the binding of H1 or H5 (Finch & Klug, 1976; Nicolini et al., 1976; Thoma & Koller, 1977). The core DNA tertiary structure is less variable. It is a superhelix of 1.75 turns, with ~80 base pairs/turn with a radius of 45 Å and a pitch of 28 Å (Finch et al., 1977).

Details of core particle DNA secondary structure (base tilt, twist, etc.) have not yet been determined, but Raman spectroscopic studies (Goodwin & Brahms, 1978) and wide-angle X-ray scattering data (Bram, 1971) indicate that it is closely similar, if not identical, with that of B-DNA. No evidence of C-DNA structure has been found. The circular dichroism (CD[1]) spectrum of core particle DNA, however, is significantly altered from that of protein-free B-DNA (Ramsay-Shaw et al., 1974; Mandel & Fasman, 1976; Whitlock & Simpson, 1976; Olins, D. E., et al., 1977; Tatchell & Van Holde, 1977; Cowman & Fasman, 1978). Both DNA–histone and histone–histone interactions are important for the maintenance of the altered DNA conformation (Olins, D. E., et al., 1977; Lilley & Tatchell, 1977; Whitlock & Simpson, 1977).

It would be of great interest if the CD data could be analyzed in terms of specific changes in DNA structure. An example of a situation in which this ability to interpret spectra would be important is the case of high molecular weight chromatin. The maximum CD ellipticity of DNA in chromatin, above 260 nm, varies from a value equal to that of core particle DNA to a higher value intermediate between that of a core DNA and protein-free DNA [see, for example, Shih & Fasman (1970), Johnson et al. (1972), Spurrier & Reeck (1976), Nicolini et al. (1976), de Murcia et al. (1978), and Fulmer & Fasman (1979a)]. Chromatin with the lower ellipticity value has been termed "native"; chromatin with the higher value has been attributed to a "disrupted" structure (de Murcia et al., 1978). These terms are not meaningful if the manner in which the DNA structure is reflected in its CD spectrum is not understood. In particular, we have recently shown that the CD spectrum of chromatin in the disrupted structure simply corresponds to the spectrum anticipated for chomatin in the "beads-on-a-string" structure commonly observed in electron micrographs (Cowman & Fasman, 1978). That spectrum was observed for high molecular weight chromatin isolated by a mild nuclease digestion procedure and studied in low ionic strength solvent (Fulmer & Fasman,

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[1] Abbreviations used: CD, circular dichroism; Tris, tris(hydroxymethyl)aminomethane; Tris–EDTA, 10 mM Tris-HCl and 0.7 mM EDTA, pH 7.5.

0006-2960/80/0419-0532$01.00/0

1979a). It is identical with the spectrum for mononucleosomes which contain not only the core particle but also the approximate DNA length and very lysine-rich histone content of a repeating unit of the chromatin structure (Cowman & Fasman, 1978).

The present study is part of an effort to expand our understanding of the CD spectrum of DNA in nucleoprotein. In a previous paper (Cowman & Fasman, 1978), the CD properties of four preparation types of mononucleosomes were described. The preparations differed in both total average DNA length and content of very lysine-rich histones. The CD spectra were found to be strongly dependent on those differences. In the present study, the separate influences of these factors are investigated. Quantitative analysis of the CD data in terms of structural details for both core DNA and linker DNA is supported by further characterization via thermal denaturation analysis. The resultant conclusion is that removal of all H1 and H5 from mononucleosomes containing linker DNA leads to a destabilization and structural alteration of some core DNA. The previously proposed hypothesis (Cowman & Fasman, 1978) that the CD spectra reflect DNA tertiary structure is expanded and shown to correlate CD spectra with data obtained by other physical and biochemical methods.

Methods

Isolation of Mononucleosomes. The isolation of mononucleosomes with different average DNA lengths has previously been described (Cowman & Fasman, 1978). The core particle mononucleosomes (preparation type I) were isolated by the method of Tatchell & Van Holde (1977), which involves micrococcal nuclease (EC 3.1.4.7, Worthington) digestion of H1- and H5-depleted chicken erythrocyte nuclei. Mononucleosomes with longer average DNA lengths were isolated by an adaptation of the method of Ramsay-Shaw et al. (1976), as described elsewhere (Cowman & Fasman, 1978). All mononucleosome preparations were dialyzed against 0.25 mM EDTA, pH 7.0, for 2 days prior to study. These preparations are hereafter referred to as "unperturbed mononucleosomes".

Isolation of H1- and H5-Depleted Mononucleosomes. Purified mononucleosomes containing ~1 mg of DNA in 4 to 5 mL of 10 mM Tris-HCl and 0.7 mM EDTA, pH 7.5 (Tris–EDTA), were dialyzed for 2 days against 0.6 M NaCl, 10 mM Tris-HCl, and 0.1 mM EDTA, pH 8.0, to selectively dissociate the histones H1 and H5 (Ohlenbusch et al., 1967). The dissociated histones were removed from the samples by chromatography on a Bio-Gel A-15m (100–200 mesh) column, 1.2 × 106 cm, equilibrated with the same solvent. A continuous flow exponential gradient dialysis procedure (Carroll, 1971; Fulmer & Fasman, 1979a) was employed to lower the ionic strength in the solution of H1- and H5-depleted mononucleosomes, using the following parameters: V_1 = 500 mL; C_1 = 0.6 M NaCl, 10 mM Tris-HCl, and 0.1 mM EDTA, pH 8.0; V_2 = 2000 mL; C_2 = 0.25 mM EDTA, pH 7.0; flow rate ~50 mL/h.

Upon completion of the gradient, the samples were dialyzed for 2 days against 0.25 mM EDTA, pH 7.0. These preparations are hereafter referred to as "H1- and H5-depleted mononucleosomes".

Isolation of Mononucleosome DNA. For electrophoretic analysis of DNA molecular weight, DNA was extracted from isolated mononucleosomes by a modification of the method of Marmur (1961), as previously described (Cowman & Fasman, 1978).

The larger scale isolation of mononucleosome DNA for spectroscopic and thermal denaturation analyses was performed essentially by the method of Britten et al. (1974). The mononucleosome sample was dialyzed overnight into 0.1 M NaCl and 0.05 M EDTA, pH 7.8. Protein was digested by incubation at 37 °C for 1.5 h in the presence of 100 μg/mL proteinase K (Merck) and 0.5% sodium dodecyl sulfate. The sample was made 1 M in $NaClO_4$ and subjected to further deproteinization by shaking with chloroform–isoamyl alcohol (24:1) and phenol as described by Britten et al. (1974). DNA was precipitated by addition of 2 volumes of 95% ethanol, collected by centrifugation, and redissolved in 0.015 M NaCl and 0.0015 M sodium citrate, pH 7.0. Aliquots were dialyzed into 0.25 mM EDTA, pH 7.0, for analysis.

Gel Electrophoresis of DNA. DNA was characterized with respect to molecular weight by electrophoresis on 4% polyacrylamide slab gels by the method of Maniatis et al. (1975). The gels had an acrylamide/*N,N'*-methylenebis(acrylamide) ratio of 4:0.13 and were cast in 0.1 M Tris–borate, pH 8.3, and 0.001 M EDTA (Peacock & Dingman, 1967). The reservoir buffer was identical with the gel buffer. The 11 × 15 × 0.3 cm gels were electrophoresed at 20 mA for 20 min and then at 40 mA for 1.25 h. Gels were stained with ethidium bromide at 0.4 μg/mL in H_2O and photographed under UV illumination. DNA molecular weight distribution and purity were also characterized by electrophoresis on 6% polyacrylamide tube gels by the method of Loening (1967). The 20 × 0.6 cm tube gels had an acrylamide/*N,N'*-methylenebis(acrylamide) ratio of 6:0.15 and were cast in 0.04 M Tris–acetate, 0.02 M sodium acetate, and 0.002 M EDTA, pH 7.8. Electrophoresis was at 5 mA/gel for 6–8 h. The gels were stained in the absence of light with 0.005% Stains-All (Eastman) in 50% formamide, pH 7.3, destained in water under normal room illumination, and scanned at 600 nm. The scans were resolved into component contributions with a Du Pont 310 curve resolver.

For calibration purposes, *Hin*cII–*Hin*dIII (New England Bio Labs) restriction enzyme fragments of λ-DNA (Miles) were coelectrophoresed with all samples. Determination of the calibration fragment sizes is described elsewhere (Fulmer & Fasman, 1979a).

Gel Electrophoresis of Histones. Electrophoresis of histones was performed on sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels by the modified Laemmli (1970) method, as described by Maizel (1971). A 25-cm separating gel of 13% polyacrylamide was employed, with a 1-cm stacking gel of 3% polyacrylamide. Nucleoprotein samples were dissociated by addition of 0.2 volume of a mixture containing 10% $NaDodSO_4$, 25% 2-mercaptoethanol, 50% glycerol, 0.05% bromphenol blue, and 0.31 M Tris-HCl, pH 6.8, and heated in a boiling water bath for 2 min (Olins, A. L., et al., 1977). Approximately 10–40 μg of histone from the cooled dissociated samples was applied directly to the gels and electrophoresed at 50 V for 30 h. The gels were stained with 0.1% amido black in 20% ethanol and 7% acetic acid and diffusion destained in the same ethanol–acetic acid solvent (Olins, A. L., et al., 1977). Gels were scanned at 570 nm. Areas of the stained histone bands were determined with a Du Pont 310 curve resolver.

Spectroscopic Analysis. Absorption spectra were recorded on a Cary 14 spectrophotometer at room temperature (23 °C). Circular dichroism spectra were recorded at 23 °C with a Cary 60 with a 6001 circular dichroism accessory, as previously described (Fasman et al., 1970). In all cases, the fused quartz cell lengths were either 1 or 2 cm (Optical Cell Co., Woodbine, MD). Concentrations of DNA, expressed as per mole of nucleotide residue, were determined by absorption at 258 nm [ϵ_{258} = 6800 cm^{-1} (mol of nucleotide)$^{-1}$]. The DNA concen-

Table I: Mononucleosome DNA Length Distribution

	prepn type			
	I	II	III	IV
fraction of DNA in 140-bp species[a]	1.00	0.29	0.14	0.00
fraction of DNA in >140-bp species[a]	0.00	0.71	0.86	1.00
av length of >140-bp species[b] (bp)		170	180	200
av length of total DNA[c] (bp)	140	160	175	200

[a] Data were obtained by curve resolution of scans of 6% polyacrylamide gels stained with Stains-All. bp = base pairs. Values are ±0.05. [b] Determined from mononucleosome DNA electrophoretic mobility on 4% polyacrylamide slab gels, stained with ethidium bromide, and 6% polyacrylamide tube gels, stained with Stains-All. The DNA length corresponding to the center of the larger mononucleosome DNA band was determined relative to calibrated restriction fragments of λ-DNA. Reproducibility was approximately ±5%. [c] Calculated linear combination of DNA sizes [e.g., 0.29-(140) + 0.71(170) = 161]. Rounded to nearest 5 bp.

tration in samples studied was (1.5–2.5) × 10^{-4} M in nucleotide residues. The instrument sensitivity setting was 20 mdeg for full-scale deflection. Reported spectra are averages of 3–10 spectra obtained for 2–4 different preparations of each mononucleosome type.

Thermal Denaturation. Mononucleosome and DNA samples were thermally denatured in 0.25 mM EDTA, pH 7.0, at a concentration corresponding to an absorbance at 260 nm of 0.9–1.0 in a 1-cm cell at 25 °C. The sample was warmed to room temperature and then degassed by bubbling helium through for 2 min. A jacketed 1-cm path length quartz cuvette (Hellma 1-160 B QS), with a tight-fitting Teflon stopper, was employed for the denaturation. Absorbance at 260 nm and cell temperature were continuously monitored by a Cary 14 spectrophotometer and a linear thermal probe (Mandel & Fasman, 1974) located immediately adjacent to the circulant exit. Temperature increase was controlled to a rate of ~0.2 °C/min by using a linear temperature programmer (Neslab). Computer data analysis, derivative calculation, and averaging procedures are described elsewhere (Fulmer & Fasman, 1979a). The data are presented here as the derivative of sample hyperchromicity with respect to temperature (dh/dT) and represent averages of two to four denaturation profiles. A T_m is defined as the temperature of maximum dh/dT for a particular transition. T_m values were reproducible to ±0.5–1.0 °C.

Miscellaneous. All procedures were performed at 4 °C unless otherwise noted. Solutions were prepared at room temperature by using glass-distilled water. All chemicals were reagent grade. Dialysis tubing was Spectrapor 2 (Spectrum Medical Industries) previously treated with 0.01 M $NaHCO_3$ and 0.001 M EDTA for 15 min over a steam bath followed by exhaustive rinsing in glass-distilled water. All mononucleosome preparations were subjected to spectroscopic study within 1–3 days of isolation and dialysis in 0.25 mM EDTA, pH 7.0. Longer periods of storage in this low ionic strength solvent were found to result in nonreproducible altered properties.

Results

DNA Lengths of Isolated Mononucleosomes. The molecular weight of DNA in mononucleosomes, isolated from chicken erythrocyte nuclei after treatment with micrococcal nuclease, depends on the extent of digestion. We have previously described four classes of mononucleosome preparations, differing in *average* DNA length, obtained by varying the conditions of nuclear digestion (Cowman & Fasman, 1978). The data from a greater number of similar preparations have been combined in the present study, so that there are small quantitative differences in the DNA length distribution in some preparation types.

Data on the DNA size distribution in each type of mononucleosome preparation are presented in Table I. Preparation type I consisted of mononucleosome core particles. The DNA from this species electrophoresed as a single band, corresponding to ~140 base pairs in length. Preparation types II and III contained some 140 base pair DNA and a larger amount of DNA in a heterogeneous species 170–180 base pairs in average length. DNA from preparation type IV consisted only of the larger band, which averaged 200 base pairs in length. None of the samples contained a significant amount of DNA between approximately 145 and 160 base pairs in length.

Histone Composition. The histone compositions of the different mononucleosome types are presented in Table II. In all determinations, H4 was used as an internal standard, so that the areas of the remaining stained histone bands have been expressed relative to H4. Compositional data for the unperturbed mononucleosomes have already been reported (Cowman & Fasman, 1978). The values reported herein are only slightly different, reflecting the data obtained for a larger number of similar preparations. The core histone compositions agree, within an experimental error of ~10%, with the values obtained for chicken erythrocyte chromatin or whole nuclei. The very lysine-rich histones H1 and H5, however, occurred at levels below the expected values for a complete repeating unit of chromatin. Consideration of the fraction of DNA longer than 160 base pairs in a particular preparation and the re-

Table II: Histone Composition of Chicken Erythrocyte Nuclei, Chromatin, and Isolated Mononucleosomes[a]

nucleoprotein sample	H4	H2A	H2B	H3	H5	H1
nuclei[b]	1.00	0.99	1.00	1.22	0.95	0.30
chromatin[c]	1.00	0.91	1.04	1.42	1.05	0.36
unperturbed mononucleosomes						
type I	1.00	1.02	1.08	1.34	0	0
type II	1.00	0.94	1.04	1.34	0.37	0.19
type III	1.00	0.95	1.01	1.19	0.61	0.16
type IV	1.00	0.97	1.02	1.27	0.63	0.15
H1- and H5-depleted mononucleosomes						
type II	1.00	0.93	0.93	1.31	0	0
type III	1.00	1.00	0.93	1.25	0	0
type IV	1.00	0.93	0.87	1.29	0	0

[a] Based on $NaDodSO_4$-polyacrylamide gels stained with amido black and scanned at 570 nm. Band areas are expressed relative to the area of the H4 band on the same gel. Areas were reproducible to ±0.10. [b] Cowman & Fasman (1978). [c] Fulmer & Fasman (1979a).

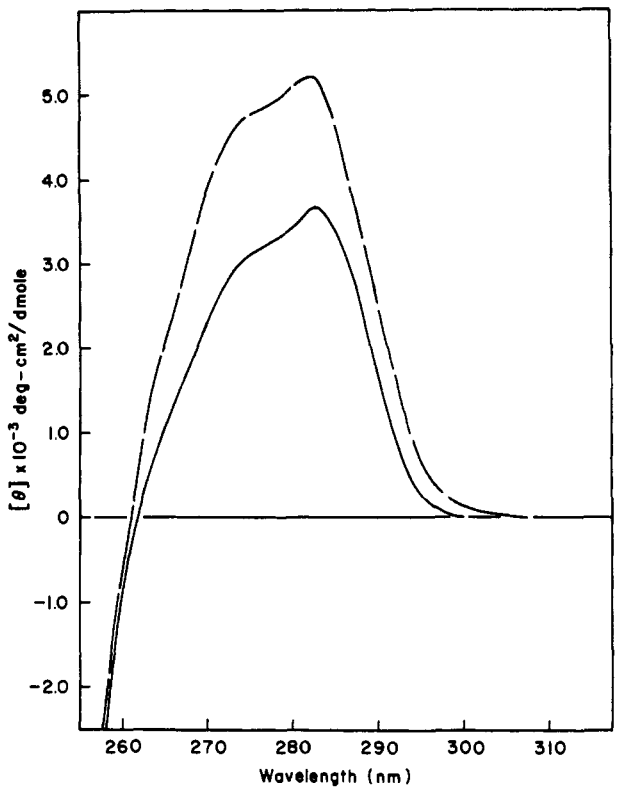

FIGURE 1: CD spectra of type IV (200 base pair average) mononucleosomes in 0.25 mM EDTA, pH 7.0. (—) Unperturbed mononucleosomes; (---) H1- and H5-depleted mononucleosomes. Ellipticity was calculated on the basis of DNA nucleotide residue concentration.

ported presence of at least two molecules of H1 and H5 per repeat unit in chicken erythrocyte chromatin (Olins, A. L., et al., 1977) leads to the conclusion that *on the average* at least one molecule of a very lysine-rich histone remains bound to each mononucleosome with attached linker DNA (Cowman & Fasman, 1978).

The core histone composition of H1- and H5-depleted mononucleosomes was identical, within experimental error, with the composition of unperturbed mononucleosomes. Analysis of the H4 to DNA ratio in both unperturbed and H1- and H5-depleted samples was performed by comparison of the stained H4 band intensity for equal applications of sample DNA content. On that basis, no core histones were lost from the samples subjected to the 0.6 M NaCl treatment and gel filtration (within ±5%).

CD of Unperturbed Mononucleosomes. The CD spectra of the unperturbed mononucleosome preparations have been previously reported (Cowman & Fasman, 1978). The data obtained for additional similar preparations have not altered the spectral parameters, which are presented in Table III. It is clear that there are significant differences in CD properties among the four preparation types. Mononucleosomes with shorter average DNA lengths exhibit lower ellipticity CD bands (140 bp, $[\theta]_{284}$ = 1900 deg cm^2 dmol^{-1}). The preparation type IV mononucleosomes, which represent a nearly complete repeating unit of the chromatin structure, exhibit a CD spectrum (200 bp, $[\theta]_{284}$ = 3700 deg cm^2 dmol^{-1}) essentially identical with that of high molecular weight chicken erythrocyte chromatin (Fulmer & Fasman, 1979a).

CD of H1- and H5-Depleted Mononucleosomes. Removal of H1 and H5 from isolated mononucleosomes has a significant effect on the CD properties of the DNA component. The CD spectra of type IV mononucleosomes before and after removal of H1 and H5 are shown in Figure 1. In both cases, the

Table III: Circular Dichroism Parameters for Chicken Erythrocyte Mononucleosomes[a]

nucleoprotein sample	$[\theta]_{max}$[b] (λ = 282-284 nm)	$[\theta]_{275}$[b]	$[\theta]_{max}/[\theta]_{275}$	λ crossover[c] (nm)
unpertrubed mononucleosomes				
type I	1900	800	2.4	270
type II	2300	1700	1.4	266
type III	3100	2600	1.2	264
type IV	3700	3100	1.2	262
H1- and H5-depleted mononucleosomes				
type II	4000	3300	1.2	262
type III	4600	3900	1.2	262
type IV	5200	4800	1.1	261

[a] Recorded in 0.25 mM EDTA, pH 7.0. [b] Molar ellipticity in units of deg cm^2 dmol^{-1}, calculated on the basis of DNA nucleotide concentration. Values are ±300 deg cm^2 dmol^{-1}. [c] To the nearest 1.0 nm.

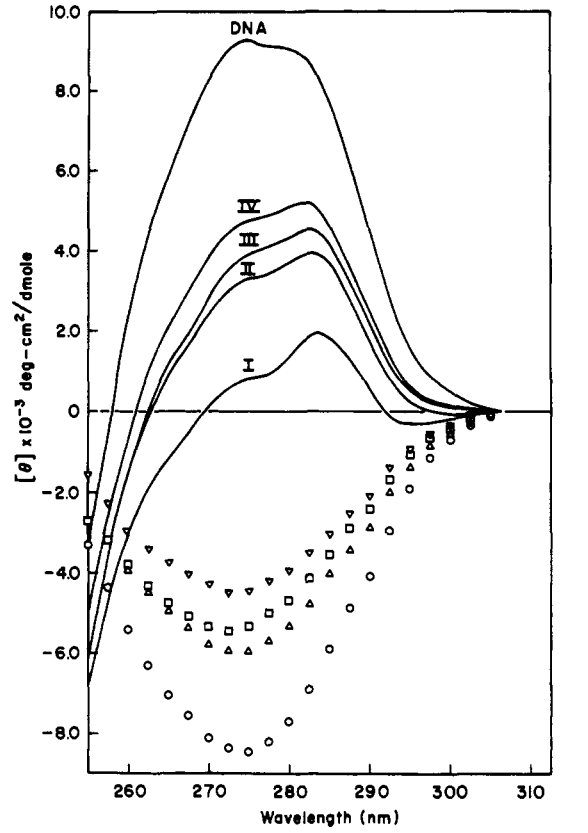

FIGURE 2: CD spectra of DNA and H1- and H5-depleted mononucleosomes and calculated difference spectra. Solvent was 0.25 mM EDTA, pH 7.0. Ellipticity was calculated on the basis of DNA nucleotide residue concentration. Curves I, II, III, and IV correspond to the four mononucleosome types, increasing in average DNA length and lacking H1 and H5. Difference spectra points were calculated by subtracting the spectrum of purified mononucleosome DNA from each of the H1- and H5-depleted mononucleosome spectra. (○) Type I minus DNA; (△) type II minus DNA; (□) type III minus DNA; (▽) type IV minus DNA.

solvent was 0.25 mM EDTA, pH 7.0. The unperturbed mononucleosomes exhibited a maximum ellipticity at 283 nm, $[\theta]_{283}$ = 3700 ± 300 deg cm^2 dmol^{-1}, and a shoulder at 275 nm, $[\theta]_{275}$ = 3100 ± 300 deg cm^2 dmol^{-1}. The H1- and H5-depleted mononucleosomes showed an overall increase in ellipticity, with $[\theta]_{282}$ = 5200 ± 300 deg cm^2 dmol^{-1} and $[\theta]_{275}$ = 4800 ± 300 deg cm^2 dmol^{-1}. The increased peak ellipticity was accompanied by a shift in the crossover (zero ellipticity)

Table IV: Transition Temperatures in Thermal Denaturation Profiles of DNA and Mononucleosomes[a]

sample	T_{m_I} (°C)	$T_{m_{II}}$ (°C)	$T_{m_{III}}$ (°C)
mononucleosome DNA[b]	41.0		
unperturbed mononucleosomes			
type I		58.5	71.5
type II	60.5	65.0	73.0
type III	58.0	62.5	73.0
type IV	58.0	c	72.0
H1- and H5-depleted mononucleosomes			
type II	51.0	c	71.0
type III	47.5	c	69.5
type IV	~45	c	68.5

[a] Thermal denaturation was performed in the solvent 0.25 mM EDTA, pH 7.0. T_m values are reported to the nearest 0.5 °C. [b] DNA isolated from type III mononucleosomes. [c] Not resolved.

point from 262 to 261 nm and a reduction in the ratio of ellipticities in the two positive bands, $[\theta]_{283}/[\theta]_{275}$. Removal of H1 and H5 from type II and type III mononucleosomes also causes an increase in ellipticity and a change in overall spectral shape, relative to the unperturbed species (Table III and Figure 2). Thus, the very lysine-rich histones appear to have a significant effect on the conformation of DNA in mononucleosomes.

Calculated CD Difference Spectra. In Figure 2, the CD spectra for core particles and H1- and H5-depleted mononucleosomes are compared to the spectrum of protein-free DNA from type III mononucleosomes. We have previously reported (Cowman & Fasman, 1978) that the CD spectrum of protein-free mononucleosome DNA is identical with the spectrum of high molecular weight DNA. Clearly, the spectra for the H1- and H5-depleted mononucleosomes exhibit both lowered intensities and altered band shapes, relative to DNA alone. The altered spectra and thus the nature of the core histone effect on DNA conformation can be investigated further by calculation of difference spectra, i.e., point-by-point subtraction of the free DNA spectrum from each mononucleosome spectrum. Figure 2 shows the results of this procedure. In each case, the difference spectrum appears to be a single negative band, centered at ~275 nm. The same effect has previously been noted for unperturbed mononucleosomes (Cowman & Fasman, 1978) and chromatin (Shih & Lake, 1972). The implication of this observation is that the DNA contribution to the mononucleosome CD spectra can be resolved into two components: a contribution identical with that of B-form DNA and a negative band at 275 nm. The intensity of the negative band contribution is related to the mononucleosome DNA length, being greatest when the DNA length is smallest.

Thermal Denaturation of H1- and H5-Depleted Mononucleosomes. The thermal denaturation of mononucleosomes, monitored by changes in absorption at 260 nm, provides information on the thermodynamic stabilization of DNA by the histones. Data for mononucleosomes lacking H1 and H5 will be considered first to analyze the effect of differing DNA lengths alone.

The derivative hyperchromicity profiles (dh/dT vs. T) of the core particle and H1- and H5-depleted mononucleosomes are presented in Figure 3. In all cases, the solvent was 0.25 mM EDTA, pH 7.0. In that solvent, protein-free mononucleosome DNA denatures with a single transition peak (T_{m_I}) at 41 °C. For the core particle mononucleosomes (type I), two transitions are observed, at 58.5 ($T_{m_{II}}$) and 71.5 °C ($T_{m_{III}}$). None of the DNA in core particles denatures below 45 °C,

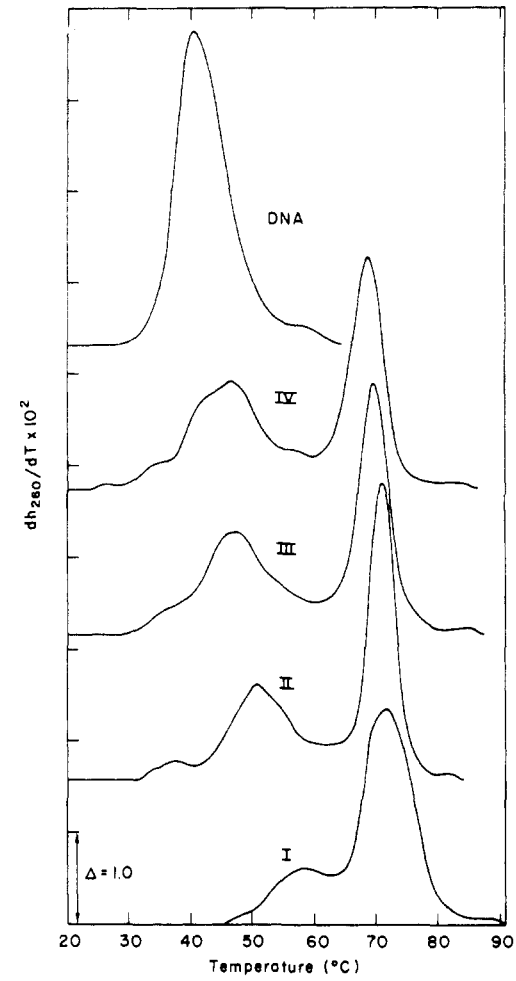

FIGURE 3: Thermal denaturation of DNA and H1- and H5-depleted mononucleosomes. The derivative of the hyperchromicity with respect to temperature, dh_{260}/dT, is plotted vs. T. Solvent was 0.25 mM EDTA, pH 7.0. Curves I, II, III, and IV correspond to the four mononucleosome types, increasing in average DNA length and lacking H1 and H5. DNA was purified from type III mononucleosomes.

indicating stabilization of the entire DNA length by the core histones. This observation is in agreement with the data of Weischet et al. (1978).

Essentially biphasic melting profiles are also observed for the H1- and H5-depleted mononucleosome preparations in which linker DNA is present. The first transition occurs at ~45–51 °C. The exact position of this peak was found to depend on the DNA length (Table IV). The preparation type IV mononucleosomes, averaging 200 base pairs in DNA length, show a first transition (T_{m_I}) at ~45 °C. The mononucleosomes with shorter average DNA lengths required a higher temperature for the first transition. T_{m_I} is tentatively assigned to the denaturation of the linker DNA regions of the mononucleosomes and possibly any destabilized core DNA.

The highest temperature melting transition observed for H1- and H5-depleted mononucleosomes occurs at 68.5–71 °C. The average DNA length also affects the exact temperature of this transition (Table IV), such that the melting temperature is lower when the linker DNA length is greater. In all cases, the temperature for this transition is lower than the corresponding temperature observed for the 140 base pair mononucleosomes. The transition also shows a smaller half-width when linker DNA is present. This transition is assigned to the most strongly bound core DNA.

The seemingly biphasic nature of the melting profiles for H1- and H5-depleted mononucleosomes (types II → IV)

Table V: Total Hyperchromicity at 260 nm upon Thermal Denaturation[a]

prepn type	unperturbed (%)	H1 and H5 depleted (%)
mononucleosomes		
I	31.5	
II	32.0	34.0
III	32.5	36.0
IV	34.0	38.0
chromatin[b]	38.0	38.0

[a] Total hyperchromicity = $100(A_{260}{}^{90°C} - A_{260}{}^{25°C})/A_{260}{}^{25°C}$; 0.25 mM EDTA, pH 7.0. [b] A. W. Fulmer and G. D. Fasman (unpublished experiments).

masks at least two additional transitions, which are detected upon close examination and curve resolution. A small peak occurs near 35–37 °C. This is below the melting temperature of bulk protein-free mononucleosome DNA and may represent a very A-T rich DNA component. There is also a broad transition at 55–60 °C. This band may correspond to the first core DNA transition observed in the 140 base pair core particles ($T_{m_{II}}$).

A further effect of DNA length on the thermal denaturation of H1- and H5-depleted mononucleosomes is a change in total hyperchromicity (Table V). The 200 base pair mononucleosomes show ~38% hyperchromicity in the DNA upon denaturation. This agrees well with the value for protein-free DNA. However, the mononucleosomes with shorter average DNA lengths show progressively lower total hyperchromicities. Core particles have a total hyperchromicity of only 31.5%.

Thermal Denaturation of Unperturbed Mononucleosomes. The derivative hyperchromicity profiles (dh/dT vs. T) of the four preparation types of unperturbed mononucleosomes are presented in Figure 4. In general, all of the profiles are similar, despite the differences in DNA length and H1 and H5 content. The strong melting band centered near 45–50 °C, noted for the H1- and H5-depleted mononucleosomes, is completely absent in every case. The histone composition data had indicated an average of at least one molecule of H1 or H5 per mononucleosome greater than 160 base pairs in DNA length (Tables I and II). *At that compositional level, all of the linker DNA is apparently stabilized against denaturation below 45 °C.*

The melting profiles of preparation type II and III mononucleosomes show three apparent bands. The middle band is not resolved in preparation type IV mononucleosomes. The profile for core particles can be fit as the sum of only two Gaussian bands. The additional melting band observed for the larger mononucleosomes should correspond to the denaturation of the linker DNA. The band which occurs at highest temperature, $T_{m_{III}}$, almost certainly is attributable to the denaturation of highly stabilized core DNA. It is not possible, however, to definitively assign the linker DNA and weakly stabilized core DNA transitions, because T_{m_I} and $T_{m_{II}}$ are poorly resolved.

The transition temperature of the first melting band is dependent on the average DNA length of mononucleosomes (Table IV). It occurs at 58 °C for the type IV mononucleosomes but at 60.5 °C for the shorter type II mononucleosomes. This behavior is similar to the temperature dependence of the first melting band in H1- and H5-depleted mononucleosomes. It may therefore be reasonable to tentatively assign T_{m_I} to the linker DNA denaturation in unperturbed mononucleosomes and $T_{m_{II}}$ to the first core DNA transition. $T_{m_{II}}$ occurs at ~62–65 °C, which might therefore indicate some stabilization of that core DNA, relative to the

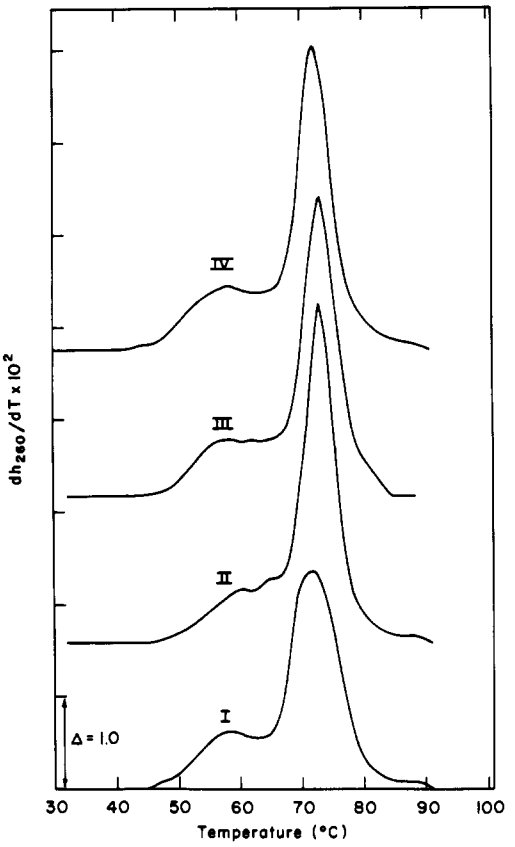

FIGURE 4: Thermal denaturation of unperturbed mononucleosomes. The derivative of the hyperchromicity with respect to temperature, dh_{260}/dT, is plotted vs. T. Solvent was 0.25 mM EDTA, pH 7.0. Curves I, II, III, and IV correspond to the four mononucleosome types, increasing in average DNA length.

case for the 140 base pair core particle mononucleosomes, where $T_{m_{II}}$ = 58.5 °C.

The highest temperature melting band, assigned to the most strongly bound core DNA, is also sensitive to the presence of H1 and H5 and linker DNA length. In core particles, $T_{m_{III}}$ occurs at 71.5 °C. For the type II and type III mononucleosomes it appears at 73 °C, reflecting the stabilizing effect of an H1- and H5-bound linker DNA segment on the core DNA denaturation. For type IV mononucleosomes, $T_{m_{III}}$ is 72 °C. It may be postulated that the longer linker DNA, which is inherently less stable in H1- and H5-depleted mononucleosomes, is less completely stabilized by H1 and H5. There may thus be opposing effects on the stabilization of the core DNA. The longer DNA length tends to destabilize the core, while the presence of H1 and H5 tends to enhance stabilization.

As was the case for H1- and H5-depleted mononucleosomes, the total hyperchromicity measured upon denaturation of unperturbed mononucleosomes depends on the average DNA length (Table V). A higher total hyperchromicity is correlated with a longer average DNA length. In addition, the hyperchromicity is affected by the presence of H1 and H5. Unperturbed mononucleosomes consistently show lower hyperchromicity than the corresponding H1- and H5-depleted samples.

Discussion

Sensitivity of CD and Thermal Denaturation Properties to Mononucleosome Composition. One purpose of the present investigation was a determination of the extent to which the CD and thermal denaturation properties of isolated mononucleosomes are dependent on the sample composition.

The effect of DNA length can be determined from a study of H1- and H5-depleted mononucleosomes, since the histone compositions of such samples are invariant. Above 260 nm, the CD spectrum of core particle mononucleosomes is of low intensity ($[\theta]_{284}$ = 1900 deg cm^2 dmol^{-1}). The same is true of H1- and H5-depleted 160 base pair mononucleosomes (Simpson, 1978). Mononucleosomes depleted of H1 and H5 but containing substantial DNA greater than 160 base pairs in length exhibit much higher ellipticities ($[\theta]_{282}$ = 4000–5200 deg cm^2 dmol^{-1}). The maximum ellipticity in each case is a function not only of the presence of linker DNA but also its length. With increasing average linker DNA length, the maximum ellipticity is raised, such that the CD spectrum becomes more like that of protein-free DNA. The thermal denaturation profiles also show DNA length dependence. When sufficiently long linker DNA is present, a low-temperature denaturation transition is observed near the temperature at which free DNA melts. This transition can be attributed to the melting of histone-free linker DNA; its exact melting temperature (T_{m_I}) depends on the linker DNA length. T_{m_I} occurs at lower temperatures as linker DNA length increases. The presence of linker DNA also has an effect on the melting temperature of the most strongly stabilized core DNA ($T_{m_{III}}$). The core DNA melts at a lower temperature as linker DNA length is increased. Finally, the total hyperchromicity observed upon denaturation increases for longer DNA. These data indicate that *the physical properties of both linker DNA and core DNA are dependent on the overall mononucleosome DNA length.*

The effect of the very lysine-rich histones H1 and H5 on mononucleosome properties can most easily be determined by comparing data for the unperturbed and the H1- and H5-depleted mononucleosomes in each DNA size type (i.e., constant DNA length). In each case, the H1- and H5-depleted species shows a higher maximum CD ellipticity than the corresponding unperturbed sample. The presence of very lysine-rich histones causes the overall DNA conformation to be less similar to protein-free DNA and more like that of core particle DNA. The thermal denaturation data indicate that H1 and H5 have a significant effect on DNA thermal stability. The total hyperchromicity is lower when H1 and H5 are present. The linker DNA in unperturbed mononucleosomes melts at a substantially higher temperature than that observed in the H1- and H5-depleted cases. *All* of the linker DNA is so stabilized, despite the observation that the "unperturbed" mononucleosomes contain less than a full complement of very lysine-rich histones. The melting temperature of core DNA is similarly elevated when H1 and H5 are present. It is not possible to discern from these data alone whether the effect on core DNA is the result of a direct interaction between H1 and H5 with core DNA or an indirect effect of linker stabilization. In either case, it is clear that *the presence of H1 and H5 affects the physical properties of both core and linker DNA.*

Because the repeating structural unit of chromatin differs in DNA length and H1 and H5 content from core particle mononucleosomes, the nonidentity of physical properties between them should to some extent be expected. A more interesting comparison can be made between chromatin and mononucleosomes averaging 200 base pairs in DNA length. The CD spectrum of the 200 base pair mononucleosomes is nearly identical with that of chromatin isolated and studied under the same conditions (Fulmer & Fasman, 1979a). Upon depletion of H1 and H5 from both species, the CD spectra remain identical. These data imply that the room temperature conformation of the DNA is the same in chromatin and 200 base pair mononucleosomes. The thermodynamic stability of the DNA, however, is not equivalent. Either in the presence or absence of H1 and H5, the melting profile of 200 base pair mononucleosomes differs from that of chromatin (Fulmer & Fasman, 1979b). It seems unlikely that the differences are the result of the slightly reduced level of very lysine-rich histones in the mononucleosomes. A recent study (McCleary and Fasman, unpublished experiments) of the melting properties of 200 base pair mononucleosomes reconstituted with increasing amounts of H1 showed that the melting properties of chromatin could not be reproduced at any ratio of H1 to nucleosome cores. Rather, a monophasic melting profile is attained at high H1 levels. Two alternative explanations for the different melting profiles of chromatin and mononucleosomes should be considered. The lower overall thermodynamic stability of DNA in mononucleosomes may reflect the presence of free (cleaved) DNA ends (Staynov, 1976). It is also possible that the melting mechanism differs in the two cases. For example, the order and the extent to which particular segments of the DNA structure (i.e., weakly histone-bound linker DNA, strongly histone-bound linker DNA, weakly stabilized core DNA, and strongly stabilized core DNA) denature may not be identical (Fulmer & Fasman, 1979b). The lower total hyperchromicity observed for unperturbed mononucleosomes relative to chromatin suggests this possibility. A difference in melting mechanisms may result in part from an alteration in the nature of H1 (H5) binding. Interaction between H1 (H5) molecules on adjacent subunits of chromatin could have a stabilizing effect not possible with isolated mononucleosomes.

Interpretation of the Compositional Dependence. A simple quantitative analysis of the CD spectra, above 260 nm, for unperturbed and H1- and H5-depleted mononucleosomes can be performed. It is based on the observation (Shih & Lake, 1972; Cowman & Fasman, 1978) that chromatin and mononucleosome spectra can be resolved into two components. One component contribution is identical with the spectrum of protein-free DNA in aqueous solution (B-DNA); the second is a negative band, centered at 275 nm, with a variable intensity. The negative band is attributed to some aspect of the DNA structure resulting from the arrangement of DNA around the histone core (Cowman & Fasman, 1978). The analysis is not dependent on the nature of the structural alteration or the mechanism by which it causes the negative band. The following assumptions are required. (1) DNA nucleotide residues in the altered structure have a set molar ellipticity contribution, which is the negative band. The residue molar ellipticity is not dependent on the number of residues involved. This contribution is in addition to the normal contribution of B-DNA. (2) Only two states are allowed for DNA nucleotide residues: altered and nonaltered. Nonaltered residues have only the normal B-DNA spectral contribution. All altered residues are spectrally equivalent to each other. Intermediate states are not allowed. (3) The CD contribution (molar ellipticity) of a nucleotide residue in the altered structure is equal to the observed contribution for core particle DNA residues. This assumes 100% participation of core particle DNA in the altered structure. (4) The spectroscopic properties of a heterogeneous preparation of mononucleosomes represent the simple weighted average of the properties of its constituent species and may be considered equivalent to the properties of its average species.

The results of the CD analysis are given in Table VI. Two striking correlations are found. *All unperturbed samples*

Table VI: Quantitative Analysis of Calculated Circular Dichroism Difference Spectra

nucleoprotein sample	av DNA length[a] (= L) (bp)	CD difference band intensity[b] ($[\theta]_{275}^{diff} = [\theta]_{275}^{sample} - [\theta]_{275}^{DNA}$)	fraction of DNA contributing to CD difference band[c] (= F)	no. of DNA base pairs contributing (= $F \cdot L$)
unperturbed mononucleosomes				
type I	140	−8500	1.00	140
type II	160	−7600	0.89	142
type III	175	−6700	0.79	138
type IV	200	−6200	0.73	146
chromatin[d]	210	−5600	0.66	139
H1- and H5-depleted mononucleosomes				
type II	160	−6000	0.71	114
type III	175	−5400	0.64	112
type IV	200	−4500	0.53	106
H1- and H5-depleted chromatin[d]	210	−4600	0.54	113

[a] From Table I. bp = base pairs. [b] From Figure 2 and Table III in units of deg cm^2 dmol^{-1}. Values are ±300 deg cm^2 dmol^{-1}. [c] Equal to ratio of sample CD difference band intensity to core particle CD difference band intensity. [d] From Fulmer & Fasman (1979a).

(chromatin and mononucleosomes) appear to have ~140 base pairs of DNA in the altered structure, regardless of the total average DNA length. This result is also independent of the variable levels at which H1 and H5 are found in the different mononucleosome preparations. Apparently, only one molecule of H1 or H5 (the minimum compositional level in the unperturbed samples) is required to affect the DNA structure, although approximately two molecules are present per repeat unit of chicken erythrocyte chromatin (Olins, A. L., et al., 1977). The second correlation is that *all H1- and H5-depleted samples, where DNA greater than 160 base pairs in length is present, have only ~110 base pairs of DNA in the altered form.* The quantitative analysis leads to the conclusion that 30 base pairs of DNA change from the altered form to the nonaltered form when H1 and H5 are removed. Moreover, those 30 base pairs must originate in the core structure if the initially altered 140 base pairs correspond directly to the core particle structure. The disruption of some core DNA structure upon removal of H1 and H5 apparently depends on a threshold length of DNA, since no similar effect is noted for the 160 base pair chromatosome (Simpson, 1978). Our preparation type II "averaging" 160 base pairs in length actually contains very little of the chromatosome species.

An interesting comparison between the CD data and the thermal denaturation data can be made on the basis of the CD analysis. Resolution of melting profiles into component bands allows determination of the fraction of the total DNA which denatures in each transition (Li, 1978). The DNA length dependence of mononucleosome T_m values and the heterogeneity of the larger mononucleosome preparations with respect to DNA length preclude accurate resolution of the melting profiles. For chromatin and core particles, however, this resolution can be performed. The areas of the two highest temperature melting bands for unperturbed chromatin correspond to approximately 48 and 107 base pairs, respectively (Fulmer & Fasman, 1979b). It is possible to speculate that the 107 most highly stabilized base pairs are identifiable with the ~110 base pairs which the CD analysis indicates remain in an altered structure upon H1 and H5 removal. In addition, the total DNA in the two highest temperature transitions (~155 base pairs) corresponds very roughly to the ~140 base pairs of DNA which are indicated by CD analysis to exist in the altered structure when H1 and H5 are present. For H1- and H5-depleted chromatin, the sum of the areas for the two highest temperature melting bands is ~100 base pairs (Fulmer & Fasman, 1979b). Again, this result can be correlated with the CD analysis, wherein ~110 base pairs of DNA appear to retain the altered structure. The melting profile of core particles can be similarly examined. The two component melting bands correspond to the denaturation of 40 and 100 base pairs, respectively (Weischet et al., 1978). The more highly stabilized 100 base pairs may be identifiable with the DNA segment which remains in the altered structure, even when sufficiently long linker DNA lacking H1 and H5 is present.

The combination of CD and thermal denaturation data thus indicates that the core DNA consists of two differentially stabilized regions. When more than 20 base pairs of linker DNA are present but H1 and H5 are removed, only the more highly stabilized DNA segment (~100–110 base pairs) remains in an altered structure. In the absence of either linker DNA or the very lysine-rich histones (i.e., core particles), the less highly stabilized DNA retains the altered structure, according to the basic assumption of the CD analysis. The implication is that long linker DNA which is not histone-stabilized contributes to the destabilization of a segment of core DNA. This conclusion may be related to the observation that the T_m of the main core DNA melting band ($T_{m_{III}}$) for H1- and H5-depleted mononucleosomes is dependent on the length of the linker DNA. Indeed, the $T_{m_{III}}$ value for H1- and H5-depleted mononucleosomes with linker DNA was in all cases studied, herein, lower than the $T_{m_{III}}$ for core particle DNA.

Dependence on Factors Other Than Composition. The CD and thermal denaturation data which have been compared in this study were obtained in the same solvent, 0.25 mM EDTA. Because the solvent can have a significant effect on the physical properties of mononucleosomes and chromatin (Gordon et al., 1979; Dieterich et al., 1979; Wu et al., 1979), it is imperative that a single solvent be employed. The very low ionic strength of 0.25 mM EDTA solution may lead to a structural destabilization, wherein only the more strongly histone-bound segments, as determined in the thermal denaturation analysis, adopt the altered structure detected by CD analysis. It is likely that under other conditions, the correlations between CD and thermal denaturation data may not hold.

The procedure by which a sample is isolated and purified can also affect the physical properties. The chromatin [Fulmer & Fasman (1979a) and unpublished experiments] with which the mononucleosomes have been compared was isolated by a procedure essentially identical with that employed in the isolation of the linker DNA-containing mononucleosomes.

Chromatin isolated by other procedures can exhibit CD ellipticity values essentially like that of core particle mononucleosomes ($[\theta]_{284} \approx 2000$ deg cm^2 dmol^{-1}) [see, for example, de Murcia et al. (1978)]. These data can be explained by the CD analysis procedure to represent a situation in which essentially all of the DNA, including linker DNA, adopts the same altered structure as core DNA.

A more detailed discussion of the CD dependence on factors other than composition is given elsewhere (Fasman & Cowman, 1978; Cowman & Fasman, 1978).

Defining the Altered Structure. The precise nature of the structural alteration in core particle DNA which results in the appearance of a negative CD band at 275 nm is not known with certainty. In a previous paper (Cowman & Fasman, 1978), a hypothesis was suggested to explain this CD contribution in terms of the DNA tertiary structure. This hypothesis is based on the observation that the negative band is strikingly similar in band shape and position to the CD spectrum of Ψ-DNA (Jordan et al., 1972), DNA–H1 complexes (Fasman et al., 1970), DNA–poly(L-lysine) complexes (Haynes et al., 1970), and DNA in solutions of high LiCl concentration (Wolf et al., 1977). In each of these cases, the DNA is condensed into an ordered tertiary structure but retains a secondary structure similar to B-DNA (Haynes et al., 1970; Maniatis et al., 1974). The unique tertiary structure is postulated (Jordan et al., 1972) to be responsible for the unusual CD spectrum. In a similar manner, DNA wrapped such that adjacent turns of the superhelix are condensed together around the histone core, in an ordered arrangement, may also result in a tertiary structural contribution to the CD spectrum (i.e., the negative 275-nm band). The great difference in the intensity of this contribution in nucleosomes vs. Ψ-DNA may conceivably be related to the size of the ordered DNA condensate, the proportion of the DNA which is involved, the distance between adjacent helical segments, and the orientation of segments relative to one another.

Our data may be interpreted such that "altered residues" are now described as residues involved in a condensed and ordered tertiary structure. The analysis procedure leads to the conclusion that some core DNA suffers a change in tertiary structure (a decondensation) when H1 and H5 are removed from linker DNA of sufficient length.

Acknowledgments

We thank Dr. K. E. Van Holde for providing us with preprints prior to publication and Dr. A. Fulmer for many fruitful discussions. The excellent technical assistance of Angela French is gratefully acknowledged.

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Interaction of [^{14}C]Dicyclohexylcarbodiimide with Complex V (Mitochondrial Adenosine Triphosphate Synthetase Complex)†

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ABSTRACT: The kinetics of interaction of [^{14}C]dicyclohexylcarbodiimide ([^{14}C]DCCD) with complex V of the mitochondrial oxidative phosphorylation system and inhibition of its ATPase and ATP–$^{33}P_i$ exchange activities have been studied. DCCD inhibits ATP–P_i exchange several times faster than ATP hydrolysis as catalyzed by complex V and is taken up by complex V in biphasic manner. The rapid, initial phase corresponds in time to the inhibition of ATP–P_i exchange activity, while substantial inhibition of ATPase activity involves the second, slow phase of DCCD uptake as well. Dodecyl sulfate–acrylamide gels of [^{14}C]DCCD-treated complex V show only a single, narrow protein band covalently labeled. The labeled polypeptide is extractable into acidified chloroform–methanol (2:1), and its capacity for labeling is 6 to 7 nmol of DCCD per mg of complex V protein. There is a linear correlation between [^{14}C]DCCD labeling of this band and inhibition of ATP–P_i exchange and ATPase activities, at least up to 80–85% activity inhibition. Extrapolation to zero activity indicates that complete inhibition of ATP–P_i exchange and ATPase activities corresponds, respectively, to labeling of about one-sixth and one-half of the total specific DCCD binding sites of complex V. Covalent interaction of [^{14}C]DCCD with the DCCD-binding polypeptide of complex V is inhibited by prior treatment of the enzyme with rutamycin, venturicidin, carbonyl cyanide *m*-chlorophenylhydrazone, dibutyl(chloromethyl)tin chloride, diamide, and *N*-phenyl-*N'*-*n*-nonylthiourea (an uncoupler). Among these ATPase inhibitors and uncouplers (except diamide which inhibits neither the ATPase nor the ATP–P_i exchange activities of complex V), venturicidin and dibutyl(chloromethyl)tin chloride were found at the concentrations used to inhibit covalent DCCD binding completely.

Beechey and co-workers (Beechey et al., 1966, 1967) discovered that dicyclohexylcarbodiimide is a specific inhibitor of ATP synthesis and hydrolysis in mammalian mitochondria. Subsequent studies showed that, similar to oligomycin, DCCD[1] reacts with the membrane sector (F_o) of the mitochondrial ATPase complex (Hollaway et al., 1966), that the binding involves a low molecular weight (~8000) hydrophobic proteolipid extractable with chloroform–methanol (2:1) (Cattell et al., 1970, 1971), and that a similar DCCD-binding protein is present in chloroplasts (Nelson et al., 1977; Sigrist-Nelson et al., 1978; Sigrist-Nelson & Azzi, 1979), *Neurospora* and yeast mitochondria (Sebald et al., 1979), *Escherichia coli* plasma membrane (Fillingame, 1975, 1976; Altendorf, 1977), and the thermophilic bacterium PS3 (Sone et al., 1979). Experiments suggesting that oligomycin and DCCD bind to the same or interacting sites in mitochondria have been published (Enns & Criddle, 1977), and the isolated proteolipid from yeast has been shown to increase the proton conductance of phospholipid membranes in an oligomycin-sensitive manner (Criddle et al., 1977). The amino acid sequences of the DCCD-binding proteins isolated from *Neurospora crassa*, *Saccharomyces cerevisiae*, and *E. coli* have been published (Sebald & Wachter, 1978). The DCCD-binding residue has been determined to be a glutamic acid at position 65 in the *Neurospora* and the same corresponding position in the yeast proteins and an aspartic acid at the same relative position in the *E. coli* protein (Sebald & Wachter, 1978). The DCCD-binding residue is located in the above three proteins in the center of a hydrophobic sequence of amino acids. In oligomycin-resistant mutants of *Neurospora* and yeast, single amino acid substitutions have been identified, which are four to six residues removed from the site of DCCD binding (Sebald & Wachter, 1978).

In spite of these important developments on the molecular and functional properties of DCCD-binding proteins (mainly from microorganisms) and the site of DCCD interaction in these molecules, very little has been done regarding the kinetics of DCCD binding to submitochondrial particles and ATPase complex preparations and regarding the relationship of binding to the kinetics of inhibition of ATP synthesis and hydrolysis. In most instances, binding studies involving radioactive DCCD have been performed after prolonged incubation of the enzyme

† From the Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received September 11, 1979.* Supported by U.S. Public Health Service Grant AM 08126 and National Science Foundation Grant PCM-7826790.
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[1] Abbreviations used: DCCD, dicyclohexylcarbodiimide; DNP, 2,4-dinitrophenol; pCMB, *p*-(chloromercuri)benzoate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; S-13, 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide; DBCT, dibutyl(chloromethyl)tin chloride; NPTU, *N*-phenyl-*N'*-*n*-nonylthiourea; NSPM, *N*-(*N*-*n*-nonyl-4-sulfamoylphenyl)maleimide; $NaDodSO_4$, sodium dodecyl sulfate; diamide, diazenedicarboxylic acid bis(dimethylamide).

0006-2960/80/0419-0541$01.00/0