Complexes of DNA with Histones f2a2 and f3. Circular Dichroism Studies†

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ABSTRACT: Two histones from calf thymus, the slightly lysine-rich histone f2a2 and the arginine-rich f3, were combined, separately, with homologous DNA. The complexes were reconstituted by means of guanidine hydrochloride gradient dialysis, and their circular dichroic (CD) spectra were examined in 0.14 *M* NaCl. The CD spectra of f2a2–DNA complexes are characterized by a positive band at 272 nm which is blue-shifted and greatly enhanced relative to the corresponding band for native DNA. This type of CD change was noted previously with f2a1–DNA and f2b–DNA complexes. In contrast, f3 histone causes only minor distortions in the DNA CD spectrum, and their character depends upon the state of the two sulfhydryl groups in f3. When the cysteines are reduced, f3–DNA complexes have a slightly increased positive band with a small blue shift; when oxidized disulfide is the predominant form, this CD band becomes slightly smaller than the native DNA value. This laboratory has now examined complexes reconstituted from DNA and all five histones of calf thymus. The sum of the CD spectra of these complexes, although very similar to the CD curve for reconstituted complexes containing whole histone, does not approximate that of chromatin; the consequence of this observation is discussed.

The histones are a set of five basic proteins which are found, in combination with DNA and other molecules, in the chromatin of higher organisms. Histones have been implicated both as structural proteins for packaging of DNA and as gross repressors of transcription (Elgin et al., 1971). There is growing evidence that the histones are clustered in specific groupings along the DNA chain.

This laboratory has been studying the interaction of calf thymus DNA with homologous histones, as part of an investigation to probe the specificity of interactions involved in chromatin structure; this work has utilized circular dichroism as the main diagnostic tool. Each type of reconstituted histone–DNA complex examined (f1–DNA, Fasman et al., 1970; f2a1–DNA, Shih and Fasman, 1971; f2b–DNA, Adler et al., 1974) yields a specific kind of CD spectrum, indicating that each histone may have a different role in stabilization of the DNA geometry in chromatin. Histone f1 depresses the positive CD band of DNA (at about 280 nm); f1–DNA complexes at low f1/DNA ratio show CD spectra similar to that of chromatin (Shih and Fasman, 1970) and at high ratios the spectra are close to that of ψ-form DNA (Jordan et al., 1972). Each of the histones, f2a1 and f2b, cause a blue-shift and a greatly increased positive CD band of DNA upon complexation, although there are slight differences between these two types of complex. When histones f1 and f2a1 are simultaneously complexed with DNA (Shih and Fasman, 1972) each histone partially inhibits the effect of the other.

The present report is concerned with the CD spectra of f2a2–DNA[1] and f3–DNA complexes, formed separately by means of Gdn·HCl[2] gradient dialysis, thus completing the study of the set of five types of calf thymus histone–DNA complexes. f2a2, a slightly lysine-rich histone, has 129 amino acids, and its sequence has recently been determined (Yeoman et al., 1972; Sautière et al., 1974). Its basic residues are distributed in the asymmetric pattern characteristic of all histones (DeLange and Smith, 1972), clustered toward the amino-terminal third of the molecule. Proton magnetic resonance studies (Bradbury et al., 1973) show that, as a consequence of this distribution, only residues 25–109 are capable of acquiring secondary structure in salt solutions. Histone f2a2 has also been examined by ^{13}C nuclear magnetic resonance (Clark et al., 1974). The kinetics and analysis of the conformational changes which occur in salt have been followed by fluorescence anisotropy and CD (D'Anna and Isenberg, 1974a). Ansevin and Brown (1971) have studied the thermal hyperchromism of f2a2–DNA (and f3–DNA) complexes.

The sequence of the arginine-rich calf thymus histone f3 (DeLange et al., 1972) contains 135 residues, with typical amino acid histone distribution (many basic residues clustered in the N-terminal part of the protein). This sequence for f3 is highly conserved in different organisms (similar to the case of f2a1, DeLange et al., 1969), with only one residue changed (Cys 96 → Ser 96) in chicken (Brandt and Von Holt, 1974), shark (Brandt et al., 1974), and carp (Hooper et al., 1973), and four differences from pea f3 (Patthy et al., 1973). Sites of in vivo acetylation (DeLange et al., 1972; Wangh et al., 1972; Ruiz-Carrillo and Allfrey, 1973) and methylation (DeLange et al., 1972; Brandt and Von Holt, 1974) are known. f3 is the only histone in somatic cells which contains cysteine, one residue in most organisms, and two in the case of higher mammals (Panyim et al., 1971). These sulfhydryl groups are reduced in interphase nuclei (Panyim et al., 1971; Sadgopal and Bonner,

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[1] f2a2 is known also as histone IIb1, LAK, or H2A; f3 is sometimes called III, ARE, or H3.

[2] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; f3-SH, histone f3 with its sulfhydryl groups in reduced state; f3-SS, f3 in oxidized form; Gdn·HCl, guanidine hydrochloride.

1970), but appear to be largely oxidized (with concomitant f3 polymerization) in metaphase chromosomes (Sadgopal and Bonner, 1970) and in sperm (Marushige and Marushige, 1974). The SH groups show abnormal pK_a and reactivity (Palau and Dabán, 1974). The same types of physical studies have been performed on f3 as with f2a2: Bradbury et al. (1973) report nuclear magnetic resonance (NMR) data showing that residues 40–135 of f3 are capable of having regular secondary structure, and D'Anna and Isenberg (1974b) have studied salt-induced conformational changes.

The present results complete the set of CD studies, from this laboratory, on the five types of reconstituted calf thymus histone–DNA complexes. f2a2–DNA complexes exhibit CD changes similar to those of f2a1–DNA and f2b–DNA. On the other hand, f3–DNA complexes display CD spectra only slightly altered from that of DNA. The nature of these changes are dependent upon the oxidation state of f3, but, in either case, f3 causes less change than does any other histone. When the cumulative effect upon the DNA CD of the five histones, at physiological ratios, is calculated, the result is not even qualitatively similar to that of the CD of native chromatin. This discrepancy suggests that the structure of chromatin is not determined solely by the sum of histone molecules bound individually to DNA, but rather, that the different types of histones interact with each other (and possibly with non-histone chromosomal proteins) and such protein complexes interact with the DNA chain. Such a concept of chromatin structure has recently been proposed, based upon accumulating evidence which indicates that chromatin is particulate in nature and contains histone clusters; for example, each of the histone pairs, f3–f2a1 and f2b–f2a2, interact strongly and specifically. (Summaries of these data are given in D'Anna and Isenberg, 1974c, and in Van Holde et al., 1974.)

Materials and Methods

Histone Preparations. Histones were extracted from calf thymus by means of the ethanol–HCl procedure of Johns et al. (1960). The f3–f2a fraction was precipitated with ethanol, and then chromatographed on Sephadex G-75 (Hnilica and Bess, 1965) in order to separate f3 from f2a. Histone f2a2 was then isolated by passing the f2a fraction through a Bio-Gel P-60 column (Hnilica and Bess, 1965). The eluent for both columns was 0.01 *M* HCl. The isolated histones were then lyophilized. The f2a2 and f3 preparations so obtained were shown to be homogeneous by the Panyim and Chalkley (1969a) method of gel electrophoresis in urea, utilizing the procedure described by Adler et al. (1975). The marker dye used was 4-benzeneazo-1-naphthylamine. The f3 sample, as isolated, consisted of 80% f3-SH, and 20% f3-SS.

Whole calf thymus histone was prepared by acid (0.2 *N* H_2SO_4) extraction (Bonner et al., 1968) of chromatin (Shih and Fasman, 1970). To prevent degradation 0.05 *M* $NaHSO_3$ was present during the isolation of the chromatin, which was subsequently sheared and extracted in 2×10^{-4} *M* NaCl (Panyim and Chalkley, 1969b).

Protein Solutions. Stock solutions of f2a2 were prepared in water, at concentrations of $3–5 \times 10^{-3}$ *M* peptide residues, adjusted to pH 7.0 with NaOH and stored frozen.

Solutions of f3, at similar concentration to the above, in either the reduced (f3-SH) or oxidized (f3-SS) form were prepared by methods given by Ruiz-Carrillo and Allfrey (1973): For oxidation, f3 in 0.1 *M* sodium phosphate, 0.1 *M* NaF, or 0.1 *M* NaCl (all at pH 7.0) was agitated by means of a wrist-action shaker in a sealed tube filled with air for 24 hr at 4°. Examination by gel electrophoresis showed similar oxidation patterns for all three solvents. The sample of f3-SS utilized for CD studies of complexes with DNA consisted of 30% f3 dimers, 32% monomeric f3 containing an intramolecular SS bond (Panyim et al., 1971), 23% oxidized oligomers (5 bands discernible), and 15% reduced f3.

Reduced f3 was obtained by dissolving 25 mg of f3 in 2.5 ml of 6 *M* urea, 0.01 *M* dithiothreitol (Calbiochem), and 0.1 *M* Tris (pH 9.0). After standing 1 hr at 40° the solution was diluted with an equal volume of water, and then trichloroacetic acid was added to a volume of 18%, causing precipitation. The f3 precipitate was collected, washed with acetone (Ruiz-Carrillo and Allfrey, 1973), and dried in vacuo over NaOH. The yield was 21 mg. Gel electrophoresis, in the presence of 0.1 *M* mercaptoethanol, showed that this sample contained 97% reduced f3 and 3% f3-SS dimers. Stock solutions of this f3-SH sample were prepared by dissolving it in water, through which nitrogen had been bubbled for 30 min, adjusting the pH to 7.0, quickly taking out aliquots for concentration analysis, adding 0.001 *M* dithiothreitol, and freezing.

Histone concentrations were determined by a modified biuret assay (Adler et al., 1971), utilizing poly-L-lysine as the standard, and color factors of 0.91 for f2a2, 0.88 for f3, and 0.92 for whole histone.

DNA and Other Reagents. Calf thymus DNA was prepared as described by Adler et al. (1971). Its average molecular weight was 9.6×10^6. DNA concentrations were measured by means of OD_{258}, using ϵ_{258} (per mole of nucleotide residue) = 6800. Sources of guanidine hydrochloride, dialysis tubing, and other chemicals are as given in Adler et al. (1975).

Histone–DNA Complexes. Complexes of DNA with f2a2, f3-SS, or whole histone were reconstituted at 4° by mixing these components, at the desired concentration and ratios, under dissociating conditions (in 2 ml of 5 *M* guanidine hydrochloride–0.002 *M* Tris), slowly stirring in the histone last. Then the Gdn·HCl concentration was gradually lowered to 0.14 *M* by means of continuous-flow linear-gradient dialysis (Adler et al., 1974) in order to anneal the complexes. The Gdn·HCl was removed by dialysis against 0.14 *M* NaCl–0.002 *M* Tris, which was the solvent used for CD measurements. All solutions were adjusted to pH 7.0. (In some experiments Tris was omitted, but the pH was maintained at 7.0; in others, with or without Tris, the pH during dialysis and in the final solution was allowed to range between 6.0 and 7.5. Neither variation affected the CD results, within experimental error. Furthermore, the presence of 0.001 *M* EDTA during dialysis and measurement did not change the results.)

Complexes of f3-SH and DNA were formed in the same manner, with the exceptions that the water used for all solutions was first saturated with N_2, and all solutions contained 0.001 *M* dithiothreitol to prevent oxidation of cysteine. Spectra of f3-SH–DNA complexes, after completion of dialysis and CD measurements, showed no trace (OD <0.01) of an absorption band at 280 nm, characteristic of oxidized dithiothreitol. (OD_{280} = 0.25 for fully oxidized 0.001 *M* dithiothreitol.)

Several samples of f3-SH–DNA complexes were reoxidized. This was accomplished by removal of dithiothreitol (dialysis vs. 0.14 *M* NaCl–0.002 *M* Tris) followed by aeration for 16 hr (wrist-action shaker).

Concentrations of DNA in the complexes were deter-

Table I: CD Parameters and Conformation of f2a2 and f3.

Histone	Solvent	Band 1[a]		Band 2		Conformation Fraction[b]		
		λ (nm)	$[\theta]^c$	λ (nm)	$[\theta]^c$	α	β	Coil
f2a2	Water	225	−3200	200	−14,100	0.08	0.26	0.65
f2a2	0.14 *M* NaF[d]	222	−6000	202	−10,700	0.15	0.31	0.54
f3-SH	Water[e]	224	−3000	201	−9,300	0.04	0.39	0.57
f3-SH	0.14 *M* NaF[d,e]	220	−5600	203	−8,900	0.09	0.39	0.52
f3-SS	Water	224	−5300	202	−11,300	0.11	0.29	0.60
f3-SS	0.14 *M* NaF[d]	220	−7200	203	−10,800	0.15	0.31	0.54

[a] Shoulder. [b] Conformation calculated by comparison to standard values of Greenfield and Fasman (1969). [c] Ellipticity values, $[\theta]$, are reported per mole of peptide residues. Average deviations in $[\theta]$ are ±700 for duplicate measurements. [d] Solution contains 0.002 *M* Tris. [e] Solution contains 0.0005 *M* dithiothreitol.

mined from OD_{258} following dissociation of the complexes by 0.25% sodium dodecyl sulfate, and were in the range of 1.0–1.3 × 10^{-4} *M* nucleotide residues. Histone/DNA ratios (*r*) are reported as moles of histone peptide residues per mole of DNA nucleotide residues, and were determined from the input concentrations.

Optical Measurements. CD spectra were recorded at 23° on a Cary 60 instrument as previously described by Adler et al. (1974). The path length for histone–DNA complexes was 1 cm. Mean residue ellipticity values, $[\theta]$, are given per mole of nucleotide residue in the complexes.

CD spectra of histone solutions were measured at a peptide residue concentration of 10^{-3} *M* in a 1-mm cell, over a wavelength range of 187–300 nm. Ultraviolet spectra were obtained on a Cary 14.

Results

Circular Dichroism of Histones f2a2 and f3. The secondary structures of the histones alone were investigated by means of CD. The CD spectral features, in water (pH 7.0 ± 0.2) and in 0.14 *M* NaF (the ionic strength used for complexes), are listed in Table I, along with estimations of conformation. In water each histone yields a CD spectrum with a negative shoulder at ~224 nm and a large negative band at ~201 nm, similar to CD curves for other histones at low salt and for denatured proteins. Such CD spectra for histones are often assumed to indicate random coil structures (D'Anna and Isenberg, 1974a,b) because of the large repulsion among positive charges present on the histone side chains. However, as is shown in Table I, if fractional amounts of secondary structure are calculated by computer comparison of the CD data with a standard set of ellipticity values based on poly-L-lysine (Greenfield and Fasman, 1969), then appreciable amounts of secondary structure, especially β sheet, appear to exist. These estimates of β form may be partially artifactual, depending upon whether the 222-nm shoulder really arises from a β-conformational component, or is instead a feature of the random coil CD spectrum of histones.

In 0.14 *M* NaF, both f2a2 and f3 acquire significant amounts (~15%) of α-helical structure. The greater fraction of α helix in oxidized f3 than in reduced f3 may be of interest. The CD spectra for f2a2, both in water and at moderate salt concentration, are in excellent agreement with those found by D'Anna and Isenberg (1974a) under similar conditions. These authors found $[\theta]_{222} = -3100$, $[\theta]_{200} = -13{,}900$ in 0.0001 *M* HCl, and $[\theta]_{222} = -6000$, $[\theta]_{204} = -10{,}900$ in 0.0033 *M* phosphate (where nearly optimal secondary structure formation occurs). The agreement for f3-SH is not as good; D'Anna and Isenberg (1974b) find a larger band at 200 nm at zero ionic strength, and a larger shoulder at 220 nm in phosphate after their slow conformational change (β-sheet formation) is complete. However, they did not examine f3 in NaCl or in NaF, so comparisons may not be completely valid. CD measurements on f2a2 and f3 (both oxidation states) in 0.01 *M* NaF yield spectra intermediate between those in water and in 0.14 *M* NaF. This observation corroborates D'Anna and Isenberg's (1974a,b) finding that the conformations of both of these histones are unusually sensitive to salt.

When calculations of conformation are performed using a set of standard values based on native proteins (Chen et al., 1972), all estimates of α-helix fraction are reduced by ~0.03, and β sheet by ~0.10 ± 0.02. This places the present estimates of α helix in good agreement with those of D'Anna and Isenberg (1974a,b), but leaves the β-sheet fractions for f2a2 and f3 much higher than those calculated by D'Anna and Isenberg (1974a,b), due largely to their choice of the random coil standard CD spectrum.

The conformation prediction method of Chou and Fasman (1974), based upon sequence, can be applied to histones f2a2 and f3. This calculation (P. Y. Chou and G. D. Fasman, unpublished data) predicts that f2a2 is capable of having 0.41 of its residues participating in α helices and 0.19 in β-chain segments; the corresponding fractions predicted for f3 are 0.39α and 0.13β. One reason why the estimation of α helix is too high for histones is that lysine residues are normally helix formers; however, portions of a protein having clusters of positively charged lysines (and arginines) are probably prevented from assuming regular secondary structure by the high charge density. Perhaps these histones cannot realize their full conformational potential under the conditions studied herein, but may do so when clustered with other histone molecules and/or combined with DNA in chromatin. In the latter case, by neutralizing their positive charge upon complexation with the DNA-phosphates, they could potentially assume the predicted conformation.

Circular Dichroism of f2a2–DNA Complexes. When complexes are formed from calf thymus f2a2 histone and homologous DNA by means of a continuous Gdn·HCl gradient, followed by removal of Gdn·HCl, striking changes are produced in the CD spectrum of DNA. (The CD of isolated DNA is not influenced by this exposure to Gdn·HCl.) It can be seen in Figure 1 that, at physiological salt concentration and a [peptide]/[nucleotide] ratio, *r*, of 1.5, the positive CD band of free DNA ($[\theta]_{278} = 8400$) is blue-shifted and enhanced to $[\theta]_{273} = 17{,}500$. The negative DNA band ($[\theta]_{245} = -10{,}100$) is decreased in magnitude to −4900, and is blue-shifted and broadened. Since the protein itself

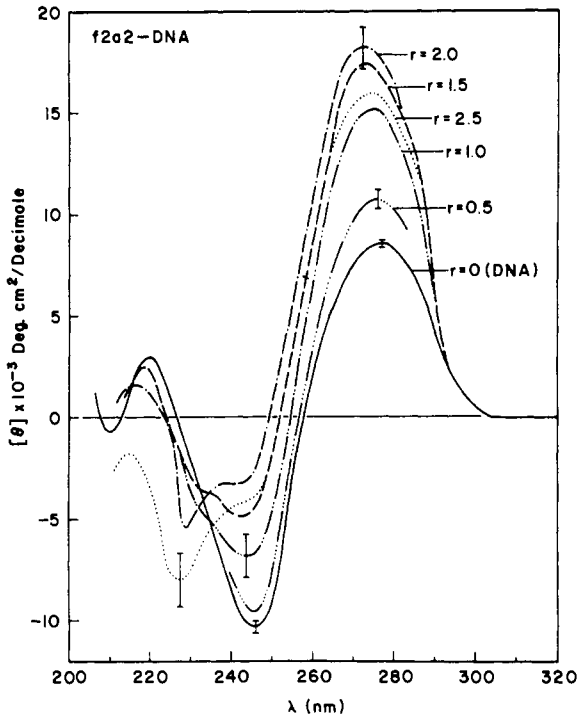

FIGURE 1: Circular dichroism spectra of complexes reconstituted from DNA and f2a2 by means of Gdn·HCl gradient dialysis. The concentration ratio of histone (moles of peptide residues) to DNA (moles of nucleotide residues) in the complexes, r, is varied from $r = 0$ (DNA alone) to $r = 2.5$. Concentration of complexes = $1.0\text{–}1.3 \times 10^{-4}$ M nucleotide residues; path length, 1 cm; temperature, 23°; solvent, 0.14 M NaCl–0.002 M Tris (pH 7.0). Error bars indicate average deviation of ellipticity data in four duplicate experiments using different complexes; reproducibility of wavelengths at extrema is typically ±1 nm.

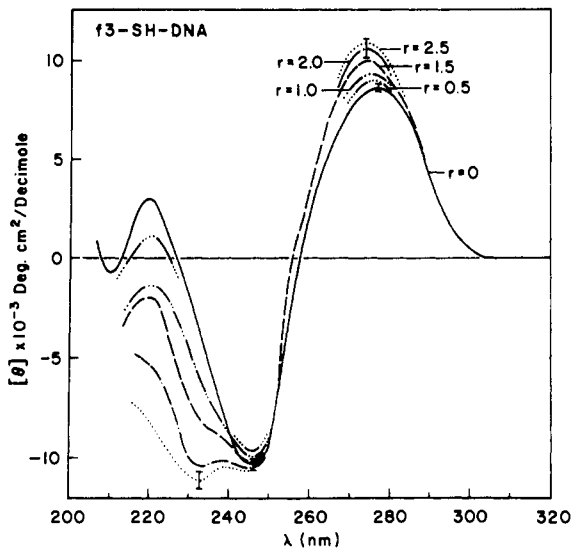

FIGURE 2: Circular dichroism spectra of complexes reconstituted with DNA and f3-SH (reduced form). Conditions are similar to those in Figure 1 except that all solutions contain 0.001 M dithiothreitol.

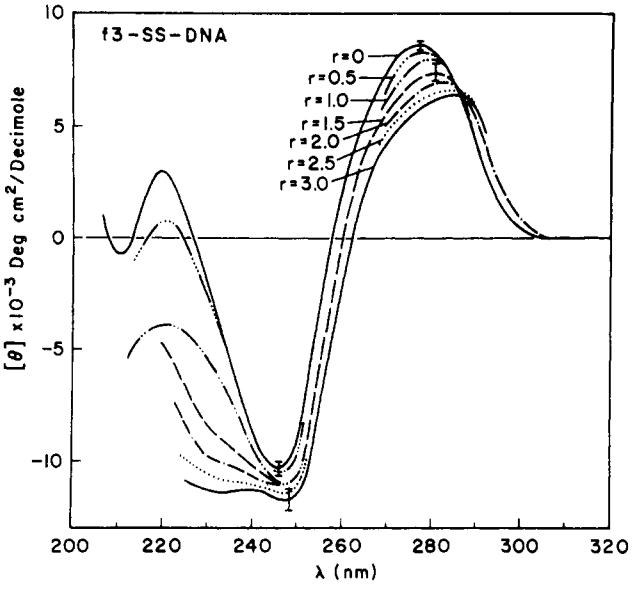

FIGURE 3: Circular dichroism spectra of complexes containing DNA and f3-SS (oxidized form). Same conditions as in Figure 1.

does not contribute to the CD spectrum at $\lambda > 244$ nm, the alterations in the DNA CD are brought about through changes in DNA conformation, coiling, or packing (folding).

These f2a2–DNA complexes at $r \leq 2.0$ display CD changes which are qualitatively very similar to those of f2a1–DNA and f2b–DNA complexes formed in Gdn·HCl gradients: for example, at $r = 1.5$, f2a1–DNA shows an average maximum CD value of $[\theta]_{273} = 16{,}800$ (Adler et al., 1975), and f2b–DNA has a peak CD of $[\theta]_{273} = 22{,}800$ (Adler et al., 1974). However, in the case of f2a2–DNA complexes, the progression of CD alteration stops at $r = 1.5\text{–}2.0$, and begins to reverse at $r = 2.5$. (This reversal is found also with f2a1–DNA complexes formed by stepwise NaCl-gradient dialysis in the presence of 5 M urea (Shih and Fasman, 1971) but not with guanidine-dialyzed f2a1–DNA.) The f2a2–DNA complexes start to precipitate at $r = 3$ (as do f3-SH–DNA complexes, but not f3-SS–DNA).

Circular Dichroism of f3–DNA Complexes. Complexes reconstituted with reduced histone f3-SH (both cysteine residues in the thiol form) are characterized by very small CD changes (Figure 2), in the same direction as alterations produced by the other arginine-rich and slightly lysine-rich histones, but of much smaller magnitude. For example, the positive band for f3-SH–DNA, $r = 1.5$, is $[\theta]_{274} = 10{,}000$. The CD spectrum near 245 nm for all f3-SH–DNA complexes is essentially that of pure DNA, and the negative trend of the spectra at $\lambda < 240$ nm reflects only the addition of protein.

On the other hand, the effect of complex formation with predominantly oxidized f3-SS (mixture of intramolecular and dimer disulfide bonds) is a progressive, small, red-shift and diminution of the positive DNA CD band (Figure 3). Although the type of change is opposite in direction to that caused by f3-SH, it is similarly small. For f3-SH–DNA at $r = 1.5$, the CD maximum is $[\theta]_{280.5} = 7400$. As in the case of reduced f3 complexes, there is very little change in the DNA negative CD band at 245 nm. The CD spectra of f3-SS–DNA complexes at high peptide-to-nucleotide ratios are similar to those produced by very low ratios ($r \leq 0.25$) of lysine-rich histone f1 (Fasman et al., 1970).

The oxidation state of the f3 cysteine residues appears to affect the structure of reconstituted complexes. This observation could be a result of different secondary or tertiary structure in some part of the f3 molecule essential for specific binding to DNA, or may arise simply from the partial dimerization of f3-SS throughout the process of complex formation. In any case, it was of interest to examine whether the structure of reconstituted f3-SH–DNA complexes

could be altered by subsequent oxidation. For this purpose, aliquots of f3-SH–DNA complexes in 0.14 *M* NaCl (duplicate sets, each ranging from r = 0.5 to 2.5) were oxidized by gentle aeration. The CD spectra of the resultant reoxidized complexes were always intermediate between f3-SH–DNA and f3-SS–DNA complexes at the same ratio. In fact, the CD spectra for all reoxidized complexes were identical within experimental error (±200 in $[\theta]$) with that of free DNA, over the wavelength range 245–320 nm; at lower wavelengths a negative protein contribution appeared. This result shows that f3-SH is bound to DNA with enough flexibility to allow the complexes, upon S–S bridge formation, to partially assume the structure of f3-SS–DNA complexes, but not completely. The mixture of disulfide bonds formed in the reconstituted complexes by oxidation may not be identical with that in the f3-SS histone sample utilized.

Circular Dichroism of Whole Histone–DNA Complexes. Complexes reconstituted from whole (unfractionated) calf thymus histone and DNA displayed an enhanced positive CD band. The peak ellipticity, $[\theta]_{274}$, maintained a plateau value of 13,000 ± 200 at r (total histone) = 1.0–2.0. The complexes precipitated at the physiological r ratio (see Discussion) of 3.0.

In order to check whether the histone isolation procedures had any effect upon complexes containing all the histones, several complexes were prepared from a mixture of all five purified histone fractions. This mixture included f1 (prepared as in Fasman et al., 1970), f2b (Adler et al., 1974), f2a1 (Adler et al., 1975), and the f3-SH and f2a2 samples used above. The relative ratios of the histones were the same as in the natural mixture (see Discussion). The results were similar to those obtained for whole histone: a plateau value of $[\theta]_{275}$ = 11,500 ± 1000 was obtained at r(total) = 1.0–2.5.

Turbidity of Complexes. All of the histone–DNA complexes used in this study showed considerable turbidity. The value of OD_{400}/OD_{258}, an empirical measure of light scattering, rose linearly with histone/DNA ratio for each type of histone. The series of f2a2–DNA, f3-SH–DNA, and reoxidized f3–DNA all displayed the same amounts of scattering; for example, at r = 1.5 all three types of complexes had OD_{400}/OD_{258} = 0.089 ± 0.003. Complexes of f3-SS–DNA consistently exhibited more scattering; at r = 1.5 the OD ratio was 0.108. This result indicates that the disulfide bridges may increase aggregation by means of cross-linking. Several complexes (of all types) at high ratios were used for measurement of CD spectra in a Fluorscat cell cell (Dorman et al., 1973). This type of cell in effect increases the angle of light detection of the photomultiplier tube of the circular dichrometer, so that most scattered light (as well as transmitted light) is detected. The CD spectra remained essentially unchanged by this measurement modification, thus showing that the observed alterations of the DNA CD spectrum are not artifacts attributable to light scattering of the complexes.

Discussion

The circular dichroic spectra of complexes reconstituted from calf thymus DNA and each of the five homologous histones, taken separately, have now been examined in this laboratory. The effect of histone f2a2 is to greatly augment the positive CD band of DNA, in a manner similar to that of f2a1 (Shih and Fasman, 1971; Adler et al., 1975) and f2b (Adler et al., 1974). f3 causes only small alterations of the DNA spectrum. Lysine-rich f1 is unique in bringing about very large changes in the opposite direction, yielding large negative ellipticities (Fasman et al., 1970).

A calculation can be made, as follows, to estimate the sum of the CD changes expected if each histone were to bind independently to DNA at physiological histone/DNA ratio. First, the calf histone composition data of Panyim and Chalkey (1969b) and the method of Adler et al. (1974) were employed to calculate the physiological r values (ratio of peptide to nucleotide) for each of the histones; these values are 0.63 for f1, 0.72 for f2b, 0.56 for f2a1, 0.55 for f2a2, and 0.59 for f3; all values are ±0.12. Next the experimental CD curves, given here and in the papers quoted above, were used to estimate the $[\theta]$ values, at any wavelength desired, corresponding to the physiological ratio for each type of histone–DNA complex. For example, $[\theta]_{282}$ = 10,600 ± 600 for f2a2–DNA at r = 0.55. Then the differences between these $[\theta]$ values for each histone–DNA type and that for DNA alone at the same wavelength were calculated, and these differences were summed. This sum represents the CD change caused by the presence of the five histones acting independently. When this sum is then added to $[\theta]$ for DNA, the result is the calculated $[\theta]$ for reconstituted nucleohistone containing noninteracting histones. The positive peak for this calculated CD spectrum is $[\theta]_{274}$ = 14,400 ± 2000; its value at 282 nm is $[\theta]_{282}$ = 13,200 ± 2000. (This result does not depend significantly upon the choice of f3-SH–DNA or f3-SS–DNA curves.) The experimental values for whole histone–DNA complexes at ratios approaching physiological are very similar ($[\theta]_{274}$ = 13,000; $[\theta]_{282}$ = 11,900) to these calculated ellipticities.

For native calf thymus chromatin (Shih and Fasman, 1970) the maximum value is $[\theta]_{282}$ = 4000 ± 300. The calculated and experimental CD spectra for nucleohistone are, therefore, not even qualitatively similar; the calculated positive CD band is much larger than that of DNA, and the measured chromatin band is much smaller. (It is of interest that chromatin appears to be irreversibly altered by guanidine treatment: when native calf thymus chromatin was dissociated in 5 *M* Gdn·HCl and then put through the linear guanidine gradient utilized for complexes, the positive CD band remained close to the value for free DNA, $[\theta]_{281}$ = 6800. However, more complete reconstitution could be achieved by treatment of chromatin with either 5 *M* urea or 2 *M* NaCl, followed by dialysis for gradual removal of the dissociating agent; in both cases $[\theta]_{280}$ = 5000.)

The discrepancy between the calculated and experimental CD spectra for chromatin is probably not mainly attributable to the presence of non-histone proteins in native chromatin. Preliminary results (A. J. Adler, L. J. Kleinsmith, and G. D. Fasman, unpublished data) show that non-histones incorporated into histone–DNA complexes moderate to a significant degree the CD distortions caused by the histones, but the non-histone protein effect is not of the type or magnitude sufficient to produce the discrepancy found here.

More likely, the sum of independent histone contributions is not a valid model for chromatin CD because the histones interact strongly with one another when bound to DNA. Shih and Fasman (1972) have shown that each of the histones, f2a1 and f1, inhibits the CD distortion caused by the other when complexed to DNA. There are physicochemical measurements showing that several pairs of histones are capable of forming specific complexes following isolation of the individual histones and mixing them together: f3 and f2a1 come together to form a tetramer containing

two molecules of each protein (D'Anna and Isenberg, 1974c,e), and f2b can form a dimer with either f2a2 (D'Anna and Isenberg, 1974c,d) or f2a1 (D'Anna and Isenberg, 1973, 1974c). (The other histones interact less strongly.) Since the histone pairs, f3–f2a1 and f2b–f2a2, can be isolated intact and characterized when chromatin is extracted by gentle methods (see references in D'Anna and Isenberg, 1974c,e), and since evidence for f2b–f2a1 interaction has been obtained by chemically cross-linking chromatin (Martinson and McCarthy, 1975) these clusters probably occur naturally on chromatin. Further evidence for this conclusion comes from the enzymatic digestion of chromatin (Weintraub and Van Lente, 1974). Several experimental approaches have recently pointed to a particulate structure for chromatin, consisting of periodic units containing DNA and sets of histone clusters (Van Holde et al., 1974, and references therein; Olins and Olins, 1974; Hewish and Burgoyne, 1973; Axel et al., 1974). In such a model for chromatin, the histones would act cooperatively, not independently, in binding to DNA and in maintaining its structure. Apparently, such cooperative behavior is not reproduced by whole histone combining with DNA during a guanidine gradient.

It is not as yet clear why the circular dichroism of DNA is altered when binding to histones occurs. Histones may induce conformational changes in the DNA double helix, or (as now seems more likely from studies of ψ-form DNA and poly-L-lysine–DNA complexes) the CD may reflect the asymmetry of a twisted, periodic packing of the DNA in the histone–DNA complexes and in chromatin. This situation may be analogous to condensed ψ-form DNA, whose CD spectrum is characterized by a large, negative band (Jordan et al., 1972), similar to that of f1–DNA complexes at high histone/DNA ratios, but which consists of DNA having the normal B-form secondary structure (Maniatis et al., 1974). Furthermore, X-ray diffraction studies (Haynes et al., 1970) of poly-L-lysine–DNA complexes, which also display inverted CD curves, indicate that DNA retains its β-form structure. The opposite (positive) sense of the CD distortion for the other histone–DNA complexes may be the consequence of a structural packing with overall twist chirality opposite to that of ψ-DNA. Complexes of DNA with statistically random copolymers of L-lysine and L-alanine (Šponar et al., 1973; Pinkston and Li, 1974) also show CD spectra with enhanced positive DNA bands at moderate salt concentration. DNA in solvent mixtures containing more than 65% ethanol also yield such spectra (Brahms and Mommaerts, 1964; Ivanov et al., 1973; Girod et al., 1973).

DNA complex formation with reduced f3 histone causes the DNA positive CD band to increase, whereas f3-SS initiates a decrease. Furthermore, f3-SS–DNA complexes are more turbid than those formed with f3-SH at the same ratios. These observations may be of interest in view of the findings that f3 is present in the SH state in interphase chromatin, but is largely oxidized in metaphase chromosomes and in sperm (see introduction). Disulfide links between f3 molecules may help to further condense DNA in chromosomes and in sperm heads, and this may be accompanied by a difference in DNA packing, as monitored by CD. Table I shows that f3-SS contains somewhat more α helix and less β sheet than f3-SH; however, it would be too speculative at present to attempt to correlate histone secondary structure with the CD of histone–DNA complexes. The f3–f2a1 tetramers which were formed in vitro contain f3 in the reduced state (D'Anna and Isenberg, 1974c), and it is not known whether oxidization of f3 disturbs the tetramer structure. Significant changes in protein secondary structure occur when histone pairs interact strongly (D'Anna and Isenberg, 1974c). If similar changes occur in histone clusters on the DNA of chromatin, and if the secondary structure of a histone affects its specific mode of binding to DNA, then it is not surprising that the circular dichroism of chromatin cannot be approximated by the sum of individual histone contributions.

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Localization of the Structural Change Induced in tRNA^{fMet} (*Escherichia coli*) by Acidic pH†

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ABSTRACT: We have compared the molecular mechanism of thermal unfolding for native tRNA^{fMet} (*Escherichia coli*) and the denatured species produced by annealing at pH 4.3. Relaxation kinetic measurements reveal that the transitions assigned to melting of TψC, anticodon, and acceptor stem helices at neutral pH remain essentially unaltered at pH 4.3, but the transition corresponding to coupled melting of tertiary structure and dihydrouridine helix is greatly affected. The T_m of this region is more than 20° higher at pH 4.3 and it has a larger enthalpy of formation than in the native state. The transition dynamics are also considerably changed. In contrast to the native structure, tRNA$^{fMet}_1$ and tRNA$^{fMet}_3$ have similar tertiary structure stabilities at pH 4.3. We conclude that the structural difference between native and acid-denatured forms is localized in the tertiary structure–dihydrouridine helix cooperative interaction region of the molecule.

The structure of tRNA molecules is frequently sensitive to their environment. The earliest examples of this were the denatured forms of tRNA^{Leu} (yeast) and tRNA^{Trp} (*Escherichia coli*) (Lindahl et al., 1966; Gartland and Sueoka, 1966; Lindahl et al., 1967), which are induced by removal of Mg^{2+} or by other changes in environmental conditions. Later, Cole et al. (1972) showed that at reduced ionic strength and without Mg^{2+}, many tRNAs take on an altered form. Furthermore, reduction of pH is also known to change tRNA structure (Bina-Stein and Crothers, 1974). Finally, it is not clear to what extent the influence of crystal form on structure may be responsible for residual disagree-

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