Interaction of Histone f2a1 Fragments with Deoxyribonucleic Acid. Circular Dichroism and Thermal Denaturation Studies†

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ABSTRACT: The glycine-arginine-rich histone, f2a1 (IV) (102 amino acids), from calf thymus was cleaved at residue 84 with cyanogen bromide. Complexes containing homologous DNA and each f2a1 fragment were reconstituted by means of Gdn · HCl gradient dialysis. The circular dichroic (CD) spectra of these complexes were all examined in 0.14 *M* NaCl. The CD spectra of the DNA–f2a1 fragment complexes did not differ appreciably from that of DNA alone in the wavelength region above 240 nm. However, intact f2a1–DNA complexes yield CD spectra which differ significantly (enhanced, blue-shifted, 273-nm band) from that of native DNA (Shih and Fasman, 1971). The small C-terminal fragment (85–102) was bound weakly to DNA under the conditions used. However, the large basic N-terminal fragment (1–83) was bound as well to DNA as was whole f2a1, but produced no CD distortion. The conformation of the N-terminal fragment, unlike intact f2a1, was not changed upon increasing the ionic strength to 0.14 *M* NaF. These results complement previous studies on f2a1 and its N-terminal CNBr fragment (Ziccardi and Schumaker, 1973). Thermal denaturation of the complexes in 2.5×10^{-4} *M* EDTA was monitored simultaneously by changes in the absorption and CD spectra. All complexes showed a thermal transition at 45° (T_{m1}), attributable to the melting of free, double-stranded DNA. In addition, f2a1–DNA and N fragment–DNA complexes displayed melting phenomena at 88 and 78° (T_{m2}), respectively, caused by the denaturation of the histone-bound DNA. This difference in T_{m2} constitutes further evidence that loss of the 18-amino-acid carboxyl end segment of f2a1 prohibits the unique type of interaction which occurs between DNA and the intact histone.

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. One of these histones, f2a1 (IV),[1] is rich in arginine and glycine, and has a sequence of 102 amino acids which is nearly identical in calf thymus (DeLange et al., 1969a) and pea seedling nuclei (DeLange et al., 1969b). When f2a1 is modified by removal of the carboxy-terminal 18 amino acids (through cyanogen bromide cleavage), the remainder of the molecule (the N-terminal fragment, N[2]) is still able to bind to T7 DNA, but not in a cooperative manner which induces folding of the DNA (Ziccardi and Schumaker, 1973).

This laboratory has been studying the interaction of calf thymus DNA with homologous histones and their fragments, as part of an investigation to probe the specificity of interactions involved in chromatin structure; this work has utilized circular dichroism (CD) as the main diagnostic tool. Each type of histone–DNA complex examined (f1–DNA, Fasman et al., 1970; f2a1–DNA, Shih and Fasman, 1971; f2b–DNA, Adler et al., 1974a) yields a specific CD spectrum, indicating that each histone may have a different role in stabilization of the geometry of DNA in chromatin. Furthermore, when either f1 (Fasman et al., 1971) or f2b (Adler et al., 1974a) are chemically bisected, and their fragments are combined with DNA, none of the complexes with fragments (or mixtures of fragments) have CD spectra characteristic of the intact histone–DNA complexes; however, in these cases the more basic histone moiety does induce a change in the DNA CD spectrum. In the present case, the N-terminal fragment of f2a1, although 84 amino acids long and carrying a high positive charge density (containing 24 of the 27 basic amino acids of f2a1), is unable to cause even a slight distortion of the DNA CD spectrum, even though N binds well to DNA. The conclusion from this result, in addition to the thermal denaturation studies of these complexes reported herein, the work of Ziccardi and Schumaker (1973), and the conserved sequence of f2a1 (DeLange et al., 1969b), is that the intact nature of this histone molecule is necessary for its proper interaction with DNA. This requirement is more stringent in the case of f2a1 than for the other histones studied to date.

Many investigations of the physical properties of f2a1 have been conducted. At very low salt concentrations the molecule is thought to exist as a random coil (Shih and Fasman, 1971; Li et al., 1972; Bradbury and Rattle, 1972) with considerable globular structure (Ziccardi and Schumaker, 1972). As the ionic strength is raised and charge repulsion reduced, f2a1 acquires considerable secondary structure, involving the section from residue 30 (approximately) to the carboxyl end of the molecule, 102 (Bradbury and Rattle, 1972; Clark et al., 1974); the N-terminal region remains randomly coiled because of its high positive charge density (DeLange et al., 1969a). Hydrodynamic and kinetic studies (Li and Isenberg, 1972; Li et al., 1972; Wickett et al., 1972;

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[1] f2a1 is known also as histone H4 in a system of nomenclature proposed at the 1974 Gordon Conference on nuclear proteins.

[2] Abbreviations used are: N and C, the N-terminal and C-terminal cleaved fragments, respectively, of f2a1; Gdn · HCl, guanidine hydrochloride.

Smerdon and Isenberg, 1974) have shown that the secondary structure (α helix and β sheet) in f2a1 involves both intramolecular and intermolecular interactions. Aggregation of f2a1 is extensive in solutions of moderate salt concentration (Edwards and Shooter, 1969; Diggle and Peacocke, 1971; Ziccardi and Schumaker, 1973), although the N-terminal CNBr fragment is incapable of this aggregation (Ziccardi and Schumaker, 1973).

It has recently been found that certain pairs of histones are capable of forming specific interactions causing association, both when mixed together in salt and, apparently, also in chromatin. f2a1 has been shown to bind to f2b in salt, in a manner which induces secondary structure (D'Anna and Isenberg, 1973), and to interact more weakly with f2a2 (D'Anna and Isenberg, 1974; Clark et al., 1974). Of great potential importance is the result that the arginine-rich histones, f2a1 and f3, are found associated together in small oligomers when histones are extracted gently from chromatin (Kornberg and Thomas, 1974; Roark et al., 1974). Furthermore, there is evidence that f2a1 and f3 are clustered together on sites in native chromatin and can be separated from other histones (Clark and Felsenfeld, 1972; Varshavsky and Georgiev, 1972). Thus, any conclusions derived from the interaction of f2a1 with DNA may have to be interpreted along with similar findings for histone f3.

Reconstituted complexes of f2a1 with DNA have been examined, under various conditions, by means of circular dichroism (Shih and Fasman, 1971; Li et al., 1971; Olins and Olins, 1971; Wagner and Vandegrift, 1972; Adler et al., 1974b), hydrodynamic methods (Ziccardi and Schumaker, 1973), and thermal denaturation monitored by ultraviolet absorption (Shih and Bonner, 1970; Ansevin and Brown, 1971). The only investigation of complexes between DNA and the N-terminal fragment of f2a1, that of Ziccardi and Schumaker (1973), has been summarized above. Although hypochromicity has frequently been utilized to examine melting transitions of histone–DNA complexes (see above) and deoxyribonucleoprotein (Ansevin et al., 1971; Li and Bonner, 1971; Li et al., 1973), the circular dichroic changes occurring during melting have been measured only for complexes of DNA with synthetic polypeptides (Mandel and Fasman, 1974) and, very recently, for deoxyribonucleoprotein (Wilhelm et al., 1974). This technique has been applied in the present study to gain more information about complexes between DNA and f2a1 or its fragments.

Materials and Methods

Histone Preparation and Cleavage. Histone fraction f2a1 was isolated from calf thymus by ethanol–HCl extraction (Johns et al., 1960) to obtain the arginine-rich histones, followed by chromatography on Sephadex G-75 and then on Bio-Gel P-60 to remove f3 and f2a2, respectively (Hnilica and Bess, 1965). The eluent for both columns was 0.01 *M* HCl. The f2a1 obtained was shown to be homogeneous by gel electrophoresis (method of Panyim and Chalkley, 1969).

The histone was cleaved at methionine residue 84 by means of cyanogen bromide (Gross, 1967), following the procedure of DeLange et al. (1968): 38 mg (0.0034 mmol) of f2a1 in 2 ml of 70% formic acid was mixed with 29 mg of CNBr (Eastman Kodak, 0.3 mmol) in 2 ml of the same solvent. After 22 hr in the dark at 23° the reaction mixture was diluted with 36 ml of water, and was lyophilized to remove CNBr and formic acid.

Chromatography of Fragments. The CNBr digest of f2a1 was subjected to exclusion chromatography on Bio-Gel P-60 (50–100 mesh, 3 × 110 cm column, eluted with 0.01 *M* HCl at 70 ml/hr), following the procedure of Ziccardi and Schumaker (1973). Two peaks were obtained, the first consisting of fragment N plus uncleaved f2a1, the second being mainly fragment C. Each peak was subsequently lyophilized and chromatographed on Sephadex G-50 (medium, 1 × 55 cm column, eluted with 0.01 *M* HCl at 15 ml/hr, 1-ml fractions collected). This procedure yielded 3 mg of electrophoretically pure fragment C, which was then lyophilized. However, the N fragment obtained was heavily contaminated with f2a1. Therefore, the preparation of N was put through a Sephadex G-100 (1 × 55 cm) eluted with 0.1 *M* NaCl plus 0.01 *M* HCl. This fractionation (Ziccardi and Schumaker, 1973) is possible because whole f2a1 aggregates in 0.1 *M* NaCl and elutes at the void volume, whereas fragment N remains monomeric. The resultant N preparation, after dialysis against 0.01 *M* HCl and lyophilization, still contained 11% f2a1. The G-100, 0.1 *M* NaCl chromatographic procedure was then repeated. The final preparation of fragment N (9 mg) was still contaminated with 6% uncleaved f2a1, as determined by gel electrophoresis.

Gel Electrophoresis. f2a1 and its fragments were examined by 15% polyacrylamide disc gel electrophoresis in 2.5 *M* urea (Panyim and Chalkley, 1969); 5–40-μg protein samples were applied in 15% sucrose. Gels were 11 cm long, and were run for 4.5 hr at 2 mA/gel. Gels were stained with Amido Black, destained electrophoretically, and scanned at 630 nm in a Zeiss microdensitometer. Integrated band areas were obtained with a Du Pont 310 curve resolver. Under the conditions used f2a1 travelled 6.8 cm, C, 9.6 cm, and N, 8.3 cm.

Protein Solutions. Stock solutions of f2a1 and its fragments were prepared in water, at concentrations of 3–5 × 10^{-3} *M* peptide residues, adjusted to pH 7.0, and stored frozen. Histone concentrations were determined by a modified biuret assay (Adler et al., 1971) utilizing poly(L-lysine) as standard and a color factor (obtained by Nessler micro-Kjeldahl analysis; Lang, 1958) of 0.92.

DNA and Other Reagents. Calf thymus DNA was prepared as described by Adler et al. (1971). Its median molecular weight was 9.6 × 10^6, determined by sedimentation velocity. DNA concentrations were measured by means of OD_{258}, using ϵ_{258} (per mole of nucleotide residue) = 6.8 × 10^3. Water was glass-distilled. Guanidine hydrochloride employed for initial mixing of histone and DNA was Research Plus absolute grade; that used for subsequent dialysis was Eastman reagent grade. Spectropore 3 dialysis tubing (molecular weight cutoff ~3500) was used for all dialyses, in order to minimize loss of the small C fragment. The tubing was boiled in 0.01 *M* $NaHCO_3$ + 0.001 *M* EDTA, rinsed, and then boiled in water before use.

Histone–DNA Complexes. Complexes of DNA with f2a1 or with its fragments (N and/or C) 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, pH 7.0), and then gradually lowering the Gdn · HCl concentration to 0.14 *M* by means of continuous-flow linear-gradient dialysis (Adler et al., 1974a) in order to anneal the complexes. The quanidine-HCl was removed by dialysis against 0.14 *M* NaCl + 0.002 *M* Tris (pH 7.0), which was the solvent used for circular dichroism measurements at room temperature (23°).

Complexes to be subjected to thermal denaturation studies were treated further by dialyzing exhaustively against 3 1-l. portions of 2.5×10^{-4} *M* EDTA (pH 7.5) at 4°.

Concentrations of DNA in the complexes were determined by ultraviolet spectroscopy following dissociation of the complexes by 0.25% sodium dodecyl sulfate, similar to the method described in Fasman et al. (1971). Concentrations of the complexes were usually $1.0–1.2 \times 10^{-4}$ *M* DNA nucleotide residues for room temperature studies, and $2.0–2.2 \times 10^{-4}$ *M* for thermal denaturation studies. Histone/DNA ratios (*r*) are reported as moles of histone (f2a1, N, or C) peptide residues per mole of DNA nucleotide residues, and were determined from the input concentrations. (In the case of C–DNA complexes, the value of *r* is merely nominal, because of dialysis losses and incomplete binding of the C fragment).

Optical Measurements. Circular dichroism spectra were recorded at 23° on a Cary 60 recording spectropolarimeter equipped with a Model 6001 CD attachment and a Cary 6003 modification which modulates the Pockels cell at 327 Hz (Adler et al., 1974a). 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.

Circular dichroism spectra of histone solutions alone (f2a1, N, and C) were measured at a peptide residue concentration of 3–4 m*M* in a 0.5-mm cell, over a wavelength range of 188–300 nm. Ultraviolet spectra were obtained on a Cary 14.

Thermal Denaturation. Complexes of DNA with f2a1, N, or C (at $r = 1.5$) were made up in 2.5×10^{-4} *M* EDTA (pH 7.5) as described above, and placed in the Cary 60 in a 1-cm jacketed cell. The temperature was slowly increased from about 10 to 102° (Mandel and Fasman, 1974) by means of a thermally programmed circulating bath (Tamson TE-3 with TP2 programmer, from Neslab, Durham, N.H.) filled with ethylene glycol. Modification of the CD dynode voltmeter allowed simultaneous recording of the ellipticity, absorbance, and temperature of the sample at a fixed wavelength (usually 280 nm) (Mandel and Fasman, 1974). At several points during denaturation, the temperature increase was stopped, and complete CD spectra were measured at fixed temperatures. The heating was then resumed. The data were processed on a PDP-10 computer and plotted on a Calcomp plotter. The extinction coefficient and the mean residue ellipticity were both plotted as a function of temperature, as well as their derivatives with respect to temperature. Only derivative plots are shown in the present paper, since these plots are more easily analyzed to determine melting temperature (T_m).

Binding Studies: Centrifugation of Complexes. The extent of binding of f2a1 and its fragments to DNA was determined by high-speed centrifugation of the complexes. Complexes of f2a1–DNA, N–DNA, and C–DNA were prepared in the usual manner (Gdn · HCl gradient dialysis). Each had a concentration of 1.0×10^{-4} *M* nucleotide residue, $r = 1.5$, in 0.14 *M* NaCl + 0.002 *M* Tris, and yielded a CD spectrum typical for its type of complex. The complexes were spun in a Spinco Model L centrifuge, utilizing a SW 50.1 rotor equipped with 0.8-ml tube adapters, at 4° for 7 hr at 40,000 rpm. Under these conditions, DNA and DNA–histone complexes were pelleted while unbound histone was found to remain completely in the supernatant. The supernatants were aspirated off, and 0.6 ml of each was dialyzed against 0.14 *M* NaF (pH 7.1) for 4 hr to remove NaCl (which interferes with absorbance measurements at low wavelength). Each sample was then bulked up to 1.0 ml with 0.14 *M* NaF, and its ultraviolet spectrum (188–300 nm) was obtained in a 1-cm cell. Histone concentrations in the supernatants were determined by utilizing extinction coefficients which had been measured at several wavelengths with f2a1, N, and C solutions whose concentrations were known from biuret assays. For example, ϵ_{195} (M^{-1} peptide, cm^{-1}) values were 7500 for f2a1, 8100 for N, and 10,000 for C.

Results

Circular Dichroism of f2a1–DNA Complexes. When complexes are formed from intact calf thymus f2a1 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 1A 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 $[\theta]_{273} = 16{,}800$. The negative DNA band ($[\theta]_{245} = -10{,}100$) is decreased in magnitude to −7000, and is blue-shifted and broadened. Since the protein itself does not contribute to the circular dichroism spectrum at $\lambda > 244$ nm (Shih and Fasman, 1971), the distortions in DNA CD are brought about through changes in DNA conformation, coiling, or interchain packing.

These f2a1–DNA complexes at $r \leq 1.5$ display circular dichroic changes similar to, but somewhat greater than, f2a1–DNA complexes which were also examined in 0.14 *M* NaCl, but which were formed by stepwise NaCl-gradient dialysis in the presence of 5 *M* urea (Shih and Fasman, 1971). (For example, a complex of f2a1–DNA, $r = 1.5$, formed by the urea method has a maximum of $[\theta]_{269} = 14{,}600$). The large differences observed by Olins and Olins (1971) between f2a1–DNA complexes formed in urea and in Gdn · HCl (by procedures somewhat different from those employed here) were not found. However, the progression of circular dichroism distortion continues beyond $r = 1.5$ in the case of Gdn · HCl-dialyzed complexes (Figure 1A), but was not found for urea-formed complexes (Shih and Fasman, 1971).

Circular Dichroism of Complexes between DNA and f2a1 Fragments. When f2a1 is cleaved with cyanogen bromide, and each resultant fragment is independently dialyzed with DNA through a Gdn · HCl gradient, neither type of reconstituted complex yields a CD spectrum significantly different from that of DNA alone in the wavelength range above 240 nm. The N-terminal fragment, N, is 83 residues long, has a high positive charge density, and (as will be shown later) binds well to DNA. However, as can be seen in Figure 1B, the N–DNA complexes formed at various *r* ratios are not conducive to circular dichroic distortion of the DNA pattern. (The small changes which occur in the positive band can be accounted for by the 6% f2a1 contamination in the N-fragment sample. For example, for N–DNA, $r = 1.5$, the experimental value of $[\theta]_{277}$ is 9700 ± 400; from extrapolation to low values of *r* of data for f2a1–DNA complexes, the f2a1 present in this N–DNA complex should cause $[\theta]_{277} = 9400$. The negative ellipticity bands at $\lambda < 240$ nm are attributable to peptide contributions.) N–DNA complexes reconstituted in the presence of urea instead of Gdn · HCl as the denaturing agent (method of Shih and Fasman, 1971) give nearly identical results (for example, at $r = 1.5$, $[\theta]_{276} = 9100$).

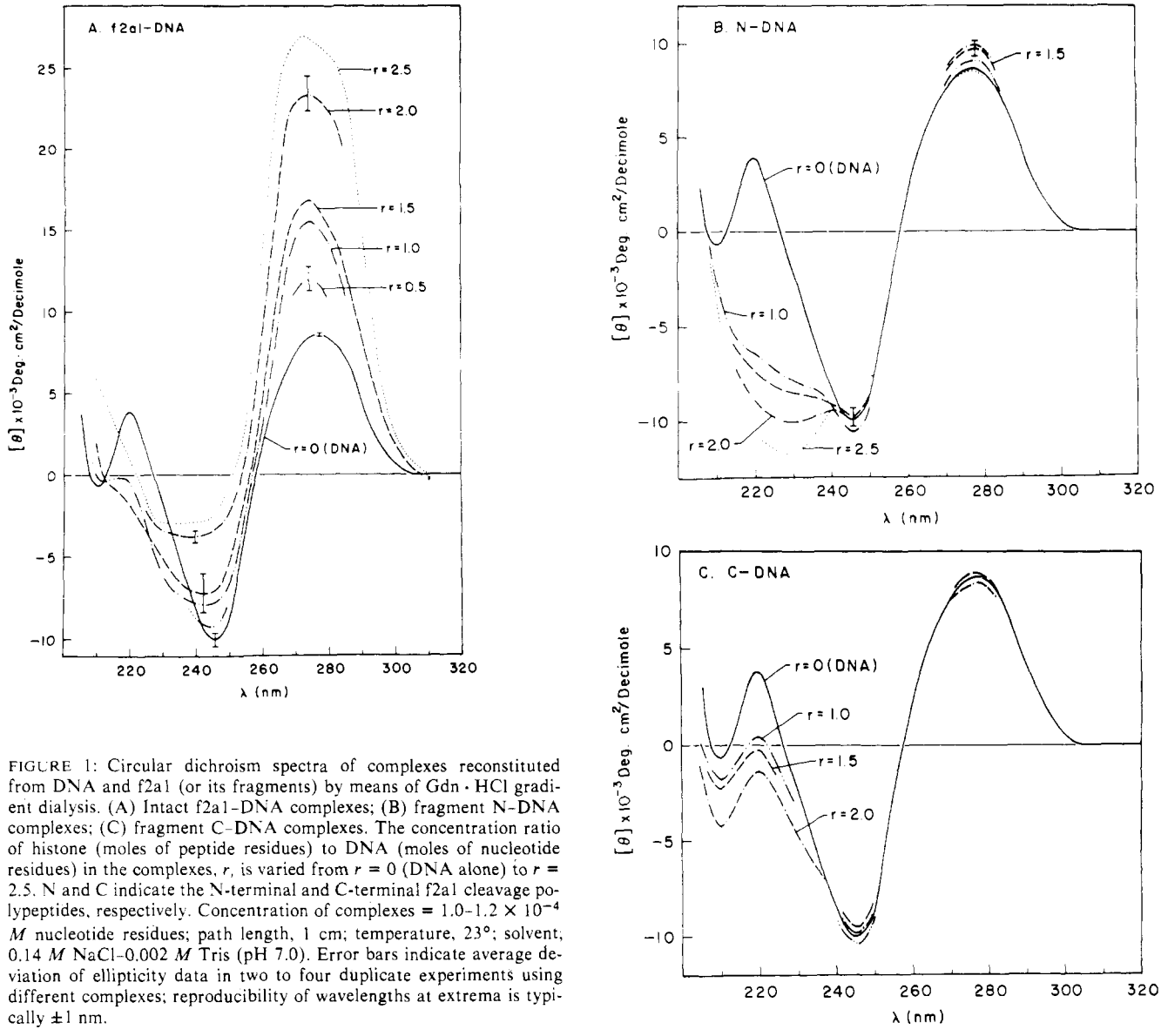

FIGURE 1: Circular dichroism spectra of complexes reconstituted from DNA and f2a1 (or its fragments) by means of Gdn · HCl gradient dialysis. (A) Intact f2a1-DNA complexes; (B) fragment N-DNA complexes; (C) fragment C-DNA complexes. The concentration ratio of histone (moles of peptide residues) to DNA (moles of nucleotide residues) in the complexes, r, is varied from $r = 0$ (DNA alone) to $r = 2.5$. N and C indicate the N-terminal and C-terminal f2a1 cleavage polypeptides, respectively. Concentration of complexes = $1.0\text{-}1.2 \times 10^{-4}$ 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 two to four duplicate experiments using different complexes; reproducibility of wavelengths at extrema is typically ±1 nm.

This failure of the large, basic, N fragment of f2a1 (which comprises over 4/5 of the molecule) to cause CD change when bound to DNA contrasts with the behavior of other histone fragments. With histones f1 (Fasman et al., 1971) and f2b (Adler et al., 1974a) the fragment which constitutes the more basic moiety of the molecule (residues 74-215 for f1 and 1-58 for f2b) binds to DNA in a manner which produces large ellipticity distortions. However, the intact f2a1 molecule is necessary to cause any CD change. This finding appears to be related to the result of Ziccardi and Schumaker (1973) that, although the N fragment binds as well as does intact f2a1 to T7 DNA in 0.1 M NaCl, the removal of 18 amino acids from the carboxyl end of histone f2a1 abolishes its ability to bind cooperatively to DNA and to cause the folding of DNA.

The small, hydrophobic, C-fragment of f2a1 is not capable of distorting the CD spectrum of DNA (Figure 1C). As will be seen later, a large fraction of C molecules is lost during dialysis, a still larger fraction does not bind to DNA, and the remainder is weakly bound and present at low [peptide]/[nucleotide] ratio (the r values given in Figure 1C are nominal imput ratios). The loss of peptide is responsible for the small negative ellipticity at $\lambda < 240$ nm. The same results were obtained with C-DNA complexes formed in the presence of urea.

Several complexes were reconstituted from DNA with mixtures of N and C fragments. Some of these mixtures contained equal peptide ratios of N and C; others were designed to have equal molar amounts of N and C molecules (with total r ranging from 0.5 to 2.5). In no instance was there any significant circular dichroism change. Thus, the two f2a1 fragments do not bind to DNA at specific sites, nor associate in such a manner as to simulate the binding of intact f2a1. The cleavage process has destroyed the ability of f2a1 to cause circular dichroism distortion.

Turbidity of Complexes. A general property of histone-DNA complexes is that, like chromatin, they form aggregates at moderate salt concentration, and therefore, scatter light. Complexes of f2a1-DNA and N-DNA are typical in this respect. Their turbidity rises linearly as r is increased, and, at any given value of r, the amount of turbidity is identical for f2a1-DNA and for N-DNA. For example, at $r = 1.5$, the value of OD_{400}/OD_{258}, an empirical measure of light scattering, is equal to 0.12 ± 0.01 for both complexes. Therefore, the ineffectiveness of the N fragment in promoting CD change in DNA cannot be attributed to artifacts

caused by simple light scattering. Complexes of C-DNA are not at all turbid, and the C fragment does not contribute to the turbidity caused by N in complexes which contain both N and C.

Binding of Fragments to DNA. The extent of binding of f2a1 and its fragments to DNA was determined by high-speed centrifugation of the same complexes, at $r = 1.5$, which were examined by CD. Ultraviolet spectroscopy of the supernatants after centrifugation showed that, for f2a1-DNA and N-DNA, very little of the histone remained unbound in the supernatant; maximum unbound amounts of protein were 5 and 3%, respectively. However, in the case of C-DNA, 46% of the polypeptide remained in the supernatant. All of the DNA was pelleted, free DNA along with that bound to histone in each complex. Centrifugation of f2a1 or its fragments alone, under identical conditions, showed that at least 99% of each protein sample remained in the supernatant. This result agrees with the centrifugal clearing times calculated from sedimentation constants for partially aggregated f2a1 at moderate salt concentration (Edwards and Shooter, 1969). Less than 2% of the DNA, when centrifuged alone, was not included in the pellet. Therefore, f2a1 can be spun out of solution, under these conditions, only when bound to DNA. Since nearly all of fragment N, as well as intact f2a1, is complexed to DNA at $r = 1.5$, the cleavage of the 18 C-terminal amino acids does not affect the ability of f2a1 to bind well to DNA. The observed CD difference between f2a1-DNA and N-DNA complexes thus reflects only variations in their internal structure.

A significant fraction of C-fragment molecules is lost by passage through dialysis tubing. Biuret concentration analysis of f2a1 and its fragments (without DNA) carried through Gdn · HCl gradient dialysis showed that, although all of the f2a1 and N molecules remained in the bags, 35 ± 5% of C was lost. An additional 5% of C molecules travel out of the bags during the 4-hr dialysis of centrifuged supernatants required to remove NaCl. When all of these losses are considered, it is seen that only 15 ± 5% of the C molecules originally mixed with DNA before Gdn · HCl dialysis is actually bound to DNA.

Complexes Formed by Direct Mixing. Attempts were made to form complexes between DNA and f2a1 (or its fragments) without use of dialysis, in order to prevent loss of the small C fragment. When the components were simply mixed and stirred by slow addition of the histone to DNA in 0.14 *M* NaCl the DNA-protein mixtures formed fibrous precipitates, unsuitable for optical studies.

On the other hand, when f2a1 (or its fragments), at $r = 1$, 1.5, or 2, was mixed with DNA in 2 *M* NaCl, followed by slow dilution with water to achieve a final solvent composition of 0.14 *M* NaCl + 0.002 *M* Tris, there was no precipitation. Mixtures of f2a1 or N fragments with DNA were turbid, indicating binding; C-DNA mixtures formed clear solutions. As in the case of gradient-dialyzed complexes, only mixtures of intact f2a1 with DNA showed any CD change. However, the nature of these changes was different; the diluted f2a1-DNA mixtures yielded progressively red-shifted and diminished long-wavelength bands. For example, at $r = 1.5$, $[\theta]_{280} = 6300$. This type of spectrum is characteristic also of f2a1-DNA complexes in the presence of calcium (Wagner and Vandegrift, 1972) or when the f2a1 contains two N^{ϵ}-acetyllysines (Adler et al., 1974b), and is not easily interpreted.

No CD change was observed from mixtures of DNA with N fragment, C fragment, or combinations of N and C, at several histone/DNA ratios. Thus, the same conclusions are obtained for mixtures (formed by dilution, with no loss of C) as are obtained for complexes formed by gradient dialysis. Only intact f2a1-DNA causes any deformation of the DNA CD spectrum. However, the f2a1-DNA complexes formed by direct mixing have not been allowed to reach the same equilibrium binding through annealing with Gdn·HCl, and their dichroic properties may be characteristic of f2a1-DNA complexes held together chiefly by nonspecific charge attraction.

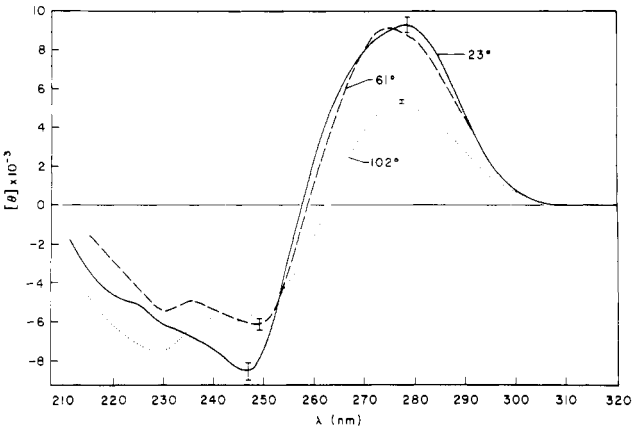

FIGURE 2: Circular dichroism spectra of intact f2a1-DNA complexes, $r = 1.5$, at various temperatures. Solvent, 2.5×10^{-4} *M* EDTA (pH 7.5); path length, 1 cm; complex concentrations = $2.0\text{-}2.2 \times 10^{-4}$ *M* nucleotide residues. Each spectrum shows the average data from two complexes used for thermal denaturation.

Thermal Denaturation. Simultaneous measurement of ellipticity and absorbance of DNA-polypeptide complexes as a function of temperature has recently been developed (Mandel and Fasman, 1974) as a sensitive probe of binding and of conformation. This method was applied herein to complexes of DNA with f2a1 or its CNBr fragments, at [peptide]/[nucleotide] ratio, $r = 1.5$. The main object of these heating experiments was to investigate whether the melting characteristics (and, hence, the binding) of N-DNA could be differentiated from those of f2a1-DNA. Complexes (and DNA controls) were formed by means of linear Gdn · HCl gradients, dialyzed into 0.14 *M* NaCl, and then dialyzed exhaustively into 2.5×10^{-4} *M* EDTA (pH 7.5). This solvent was chosen to ensure that all melting temperatures (T_m) would be well below 100°, and that ionic shielding effects would not obscure minor differences.

When the salt concentration is ≤0.01 *M*, f2a1-DNA complexes exhibit CD spectra similar to that of DNA (Shih and Fasman, 1971; Li et al., 1971). [This characteristic is shared by f1-DNA (Fasman et al., 1970) and f2b-DNA (Leffak et al., 1974) complexes.] The average CD curve for f2a1-DNA, $r = 1.5$, in EDTA at 23° is shown in Figure 2. The spectrum is conservative, and shows a low-wavelength contribution from bound histone.

As shown in Figure 2, when the temperature was raised to 61° the negative CD band at 247 nm decreased in magnitude, whereas the positive band was relatively unaffected, showing only a small blue shift. At this temperature all unbound DNA is melted, but DNA regions bound to histone remain double stranded. When the temperature was increased to 102° (thereby melting the complexed DA regions) the positive CD band decreased, but the negative band exhibited no further change. The reason for this dif-

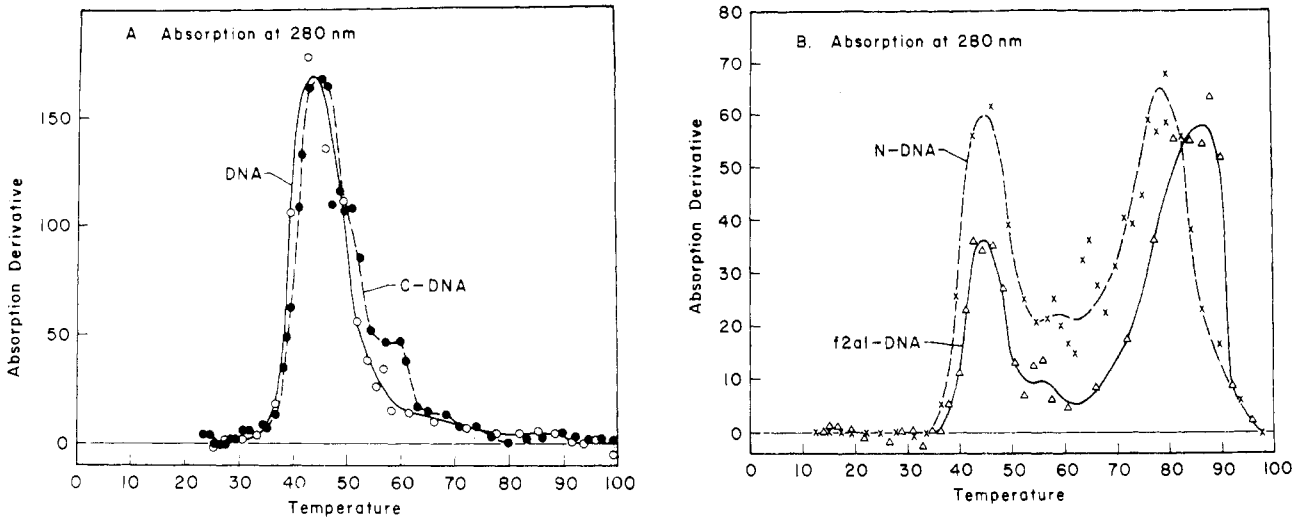

FIGURE 3: Derivative with respect to temperature of absorption thermal denaturation curves at 280 nm for complexes. (A) DNA and C-DNA complex, $r = 1.5$; (B) intact f2a1-DNA and N-DNA complexes, both at $r = 1.5$. Same experimental conditions as in Figure 2.

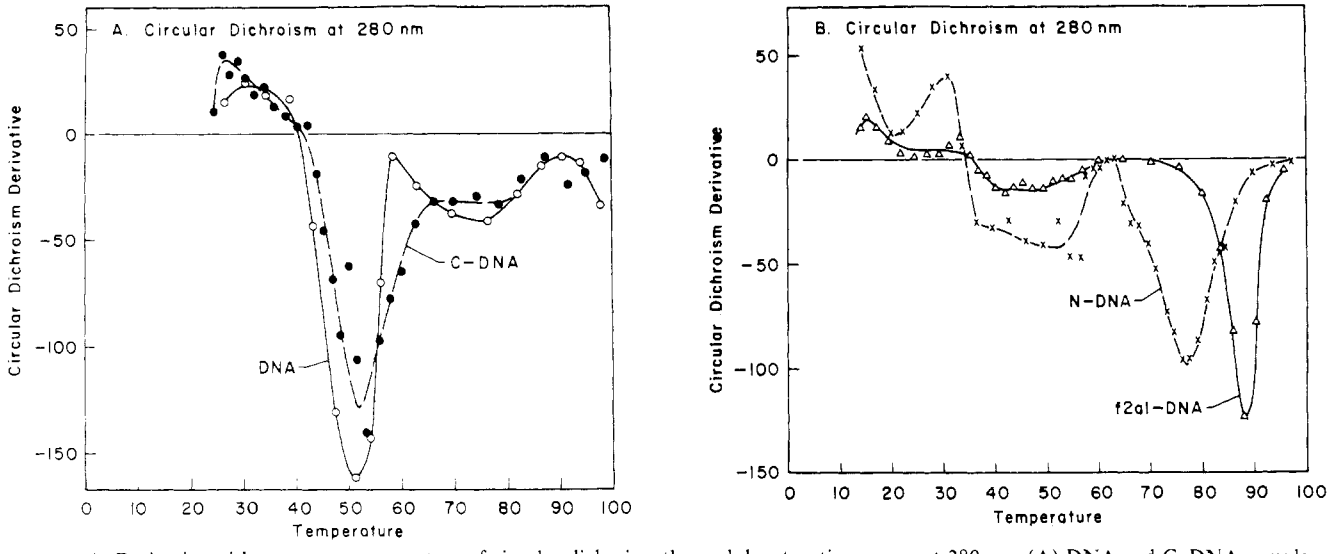

FIGURE 4: Derivative with respect to temperature of circular dichroism thermal denaturation curves at 280 nm. (A) DNA and C-DNA complex, $r = 1.5$; (B) intact f2a1-DNA and N-DNA complexes, both at $r = 1.5$. The same complexes were used as for Figure 3, and the circular dichroism data points were collected, as a function of temperature, simultaneously with the absorption data.

ferential sensitivity to heating of the two CD bands is not known. The final CD spectrum at 102° is similar to that of denatured DNA at high temperature. N-DNA complexes at these temperatures have CD spectra similar to the analogous spectra for f2a1-DNA.

The thermal denaturation method used here allows simultaneous measurement of temperature, absorption, and ellipticity of the sample at a fixed wavelength. Data were collected at 280 and 245 nm (in different experiments). Only the 280 nm results are presented here, and only derivative curves are shown (since they are easier to evaluate). Absorption derivative denaturation data at 280 nm are given in Figure 3. Each curve represents one typical experiment. The CD derivative melting plots corresponding to the same set of experiments are shown in Figure 4. Each experiment was repeated at least once with a fresh preparation of complex. Results were reproducible for the same type of complex, to the extent that the same features were always present, and the T_m values for the same thermal transition (taken as the temperatures at the extrema of the derivative curves) were reproducible to ±1.5°. The results for uncomplexed DNA are in agreement with those of Mandel and Fasman (1974).

Two major thermal transitions (Figure 3) can be discerned from absorption derivatives at 280 nm. The first, T_{m1}, at approximately 45°, is due to the loss of hypochromicity when regions of free DNA become single stranded, and is present for all samples. The second, T_{m2}, at >75°, occurs when DNA bound to (and protected by) protein is denatured, and is observed only for intact f2a1-DNA and for N-DNA (Figure 3B). These T_m values are tabulated in Table I. The small T_m band present for all samples at ~55-70° can probably be accounted for by the melting of free GC-rich DNA regions (Li et al., 1974). (The fairly large T_m absorption band at 60° for C-DNA, as seen in Figure 3A, may have a component caused by the C fragment which is loosely bound to DNA. However, since the corresponding CD melting band is no larger than that of uncomplexed DNA, and since C is only about 15% bound, with an actual peptide/nucleotide ratio of 0.2, this cannot

Table I: Thermal Denaturation Data of DNA and Complexes.[a]

Sample	T_{m1}[b] (°C)		T_{m2} (°C)		Premelt[c] (%)
	CD	Abs	CD	Abs	
DNA	51	45			5%
f2a1–DNA	~45	45	88	87	9%
N–DNA	~48	45	77	78	16%
C–DNA	52	46			5%

[a] Data taken at 280 nm in 2.5×10^{-4} *M* EDTA (pH 7.5). All complexes have nominal value of $r = 1.5$. [b] T_{m1} and T_{m2} are the major melting temperatures (see text). CD signifies data monitored by circular dichrosim; Abs, by absorption. [c] Premelt indicates the percentage increase in $[\theta]_{280}$ as the temperature is raised from 13 to 40°.

cause a large effect.) In summary, C–DNA shows an absorption denaturation curve similar to that of DNA, whereas f2a1–DNA and N–DNA exhibit an additional transition due to bound protein. This constitutes further evidence that the N fragment binds well to DNA, although no CD change is found. The melting temperature, T_{m2}, of f2a1–DNA is 9° higher than for N–DNA, indicating that the intact histone is bound more firmly to DNA than is the N fragment. A similar effect was found for the two f2b fragments by Li and Bonner (1971). The areas under the derivative curves show that a larger fraction of DNA is bound than is unbound for both N–DNA and f2a1–DNA. The turbid solutions of both of these complexes become clear during the T_{m2} transition. This causes a decrease in the observed area of T_{m2} hypochromicity relative to T_{m1}.

The circular dichroism changes, at 280 nm, which occur upon thermal denaturation are seen in Figure 4. The parameter being measured in this case is a change of asymmetry, i.e., a conformational change of the DNA, rather than loss of hypochromicity. The results are consistent with the absorbance data. DNA and C–DNA exhibit only one major CD transition, at about 50° (Figure 4A), confirming that the C–DNA sample consists mainly of free DNA. It is not apparent why T_{m1} of DNA is several degrees higher when monitored by ellipticity instead of absorption. The small CD melt at ~75° may be due to GC-rich regions. The complexes, f2a1–DNA and N–DNA, show major CD denaturations (Figure 4B) in the T_{m2} region; the T_{m2} values are consistent with those derived from absorption, with f2a1–DNA melting 11° higher than N–DNA. Examination of Figure 2 shows why the melting of free DNA regions (T_{m1}) results only in small CD changes for the complexes when monitored at 280 nm. When thermal denaturation of the complexes was followed at 245 nm, the absorption melting curves were very similar to those at 280 nm. However, the CD heating plots exhibited large bands for T_{m1} and small ones for T_{m2}, consistent with the spectra shown in Figure 2. [Some additional thermal denaturation experiments were done in 0.001 *M* citrate buffer (pH 7.0). The results were similar to those in EDTA except that in the higher ionic strength citrate the values of T_{m1} were increased about 12°, while the T_{m2} temperatures were unaffected. A similar effect was noticed by Li and Bonner (1971). The EDTA experiments are presented here because of their better resolution.]

At temperatures below T_{m1}, DNA exhibits a premelt circular dichroism change (Gennis and Cantor, 1972), in which the positive CD band increases as the temperature is raised. This behavior is also shown by DNA–polypeptide complexes (Mandel and Fasman, 1974) and, in the present case, by f2a1–DNA and N–DNA complexes. Table I summarizes the percent increases in $[\theta]_{280}$ when the temperature is raised from 13 to 40° (onset of melting). N–DNA exhibits a larger premelt than the other samples.

Circular Dichroism of f2a1 and Its Fragments Alone. As has been seen, the N-terminal fragment of f2a1 binds well to DNA but, unlike intact f2a1, induces no CD change. Furthermore, Ziccardi and Schumaker (1973) have shown that N (unlike f2a1) does not aggregate in 0.1 *M* NaCl, nor does it bind to DNA cooperatively, in a manner which causes folding. It was, therefore, of interest to examine the conformation of histone f2a1 and its fragments (especially N), under solvent conditions used for the circular dichroism studies of the complexes, in order to determine whether the protein's secondary structure might influence its mode of binding to DNA.

The circular dichroism of f2a1 and each fragment was measured in 0.005 *M* NaF (at which salt concentration Ziccardi and Schumaker (1973) found no aggregation for f2a1), in 0.14 *M* NaF (ionic strength for complexes), and in methanol (which often induces α-helix formation). Storage for 3 days at 4° did not affect the data. The CD spectral features are listed in Table II, along with estimations of conformation. The calculations of fractional amounts of

Table II: Circular Dichroism Parameters and Conformation of f2a1 and Its Fragments.

Fragment	Solvent	Band 1		Band 2		Conformation Fraction			
		λ (nm)	$[\theta]$[a]	λ (nm)	$[\theta]$[a]	α[b]	β[b]	α[c]	β[c]
f2a1	0.005 *M* NaF	220 sh[d]	−4100	202	−9,000	0.07	0.38	0.06	0.20
f2a1	0.14 *M* NaF	220 sh	−7000	206	−9,700	0.19	0.30	0.17	0.04
f2a1	95% methanol	222 sh	−4700	202	−10,800	0.08	0.33	0.03	0.21
N	0.005 *M* NaF	224 sh	−1600	198	−12,700	0.00	0.40	−0.02	0.17
N	0.14 *M* NaF	225 sh	−1600	198	−12,200	0.02	0.39	0.00	0.19
N	95% methanol	218	−8900	207	−9,700	0.20	0.34	0.20	0.12
C	0.005 *M* NaF	225 sh	−1200	198	−11,200	0.04	0.40	0.02	0.17
C	0.14 *M* NaF	224 sh	−1100	198	−11,000	0.03	0.40	0.01	0.19
C	50% methanol	223	−9900	207	−13,300	0.55	0.13	0.63	−0.87
C	95% methanol	222	−21800	208	−24,200	0.74	0.01	0.71	−0.38

[a] Ellipticity values, $[\theta]$, are reported per mole of peptide residues. Average deviations in $[\theta]$ are ±500 for duplicate measurements. [b] Conformation (fraction α helix and β sheet) calculated by comparison to standard values of Greenfield and Fasman (1969). [c] Calculated using standard values of Chen et al. (1972). [d] sh = shoulder.

secondary structure were made by computer comparison of the CD data with two standard sets of ellipticity values in the wavelength range 190–250 nm. The first standard set (Greenfield and Fasman, 1969) is based on the α-helix, β-sheet, and random coil CD curves for poly(L-lysine); this method might tend to overestimate β form in f2a1 samples when little or no α helix is present. The second set (Chen et al., 1972) uses CD values for several native proteins of known structure. The two methods yield α-helix fractions which agree well (usually within 2%) and which can be utilized for structural comparisons between samples.

In 0.005 *M* NaF, f2a1 and each of its fragments yield CD spectra with negative shoulders at ~222 nm and large negative bands at ~200 nm, similar to CD curves for other histones at low salt and for denatured proteins. The fairly large fractions of β sheet for these samples, as listed in Table II, may be partially artifactual, depending on 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. The relative lack of structure, as well as the inability of f2a1 to aggregate in low salt, can be explained by the repulsion among the many unshielded positively charged side chains. The CD spectrum of f2a1 in water (Shih and Fasman, 1971; Li et al., 1972; Adler et al., 1974b) is very similar to that in 0.005 *M* NaF.

In 0.14 *M* NaF, only intact f2a1 acquires a significant amount (about 18%) of α-helical structure. The polylysine based calculated conformation of intact f2a1 in 0.14 *M* NaF (Table II) agrees quite well with other studies: Shih and Fasman (1971) found 24% α and 36% β in 0.14 *M* NaF, and Li et al. (1972) obtained 17% α and ~30% β in ~0.01 *M* phosphate at equilibrium. This finding suggests a unique structure which can be assumed only by the whole f2a1 molecule (and not by N) in moderate salt solution, and which may be responsible for the aggregation of f2a1 and for the chirality, cooperativity, and folding of its complexes with DNA. The differences, in effects upon binding to DNA, between f2a1 and N are probably not caused solely by the slightly higher positive charge density of N (24 basic residues out of 84) than of f2a1 (27 out of 102), but may instead be the result of the need for the last 18 residues in the f2a1 molecule for maintenance of a definite structural integrity.

The C-terminal fragment does not alter its structure in 0.14 *M* NaF, although its charge density is relatively low, probably because its small chain length does not permit attainment of its full conformational potential. C was dissolved in 95% methanol in an attempt to see if α-helical structure could be induced in the fragment. The C fragment was converted to ~74% helix, thus showing that the last 18 residues may very well be structured in the intact molecule. Even 50% methanol caused a great deal of helix formation. On the other hand, methanol induced only small helicity in f2a1 or in N, possibly because charge repulsion in the large molecules is increased in a solvent of low dielectric constant.

Discussion

The main conclusion of this study is that the intact f2a1 histone molecule is necessary for the type of interaction with DNA which is fruitful in inducing circular dichroic distortion. The N-terminal CNBr fragment, although it comprises $\frac{4}{5}$ of the f2a1 molecule, and contains most of the basic residues, binds well to DNA but does not affect the DNA CD spectral properties. This situation is analogous to the cases of f1 (Fasman et al., 1971) and f2b (Adler et al., 1974a), where basic histone fragments fail to reproduce quantitatively the properties of these histone–DNA complexes. However, the case of f2a1 is more striking because the N fragment induces no CD change whatsoever. Furthermore, DNA bound to N melts at a lower temperature than does DNA bound to f2a1, and N fails to share the ability of f2a1 to acquire some α-helical structure in 0.14 *M* NaF. The complementary findings of Ziccardi and Schumaker (1973) showed that intact f2a1, but not N, aggregates in salt, binds cooperatively to DNA, and induces folding in DNA.

The C-terminal 18 amino acid peptide of f2a1 is clearly essentially for the native behavior of the histone, even though this C-terminal fragment, when cleaved from the remainder of the molecule, does not bind to DNA to a significant degree. The primary sequence of f2a1 (DeLange et al., 1969a) shows that many basic amino acids are found in the N-terminal third of the molecule, whereas the remainder of the histone is nearly neutral and contains most of the hydrophobic residues. Proton magnetic resonance studies (Bradbury and Rattle, 1972; Boublik et al., 1970) indicate that only this hydrophobic portion of f2a1 can acquire secondary structure and interact with other f2a1 molecules. Thus, it is likely that the amino-terminal part of f2a1 is its primary site of interaction with DNA, whereas the rest of the histone is available for different functions (such as binding to other protein molecules) and may not bind tightly to DNA even in the uncleaved molecule. Ziccardi and Schumaker (1973) propose a schematic mechanism for f2a1–DNA binding in which the C-terminal portion of one f2a1 molecule overlays the N-terminal part of an adjacent histone already bound to DNA. The present study confirms the importance of the carboxyl end of f2a1, but cannot distinguish between this "shingle-layering" mechanism, or a scheme in which the C-portion interacts with and protects the N-portion of the same f2a1 molecule when bound to DNA, or some other specific and necessary function for the carboxyl end.

Clusters (probably tetramers) of the arginine-rich f2a1 and f3 histones have been found to exist on chromatin (Clark and Felsenfeld, 1972; Varshavsky and Georgiev, 1972) and in histone preparations extracted from chromatin by gentle methods (Kornberg and Thomas, 1974; Roark et al., 1974). It is possible that the inter-histone forces which hold together these clusters are similar to those interactions between portions of f2a1 molecules which are responsible for the specific, CD distorting cooperative complex formation between DNA and f2a1 in this model system.

Recently evidence has been accumulating which indicates that chromatin structure is particulate in nature, consisting of periodic units containing DNA and sets of histone clusters. These particles most likely contain fewer than 200 base pairs of DNA, and may alternate with other regions of DNA which are relatively free of histone. The data for this emerging model come from studies of electron microscopy (Olins and Olins, 1974), nuclease digestion (Clark and Felsenfeld, 1971; Hewish and Burgoyne, 1973; Sahasrabuddhe and Van Holde, 1974), and from considerations of stoichiometry (Kornberg, 1974).

The role that f2a1 plays in associated clusters of both homo and hetero complexes of histones may be viewed in terms of its conformational potential. Upon addition of salt (Table II) the molecule assumes partial α-helical structure and retains a large component of β sheet. The C fragment

may contain as much as 40% β structure, independent of salt, provided that comparisons to the standard CD curves used are valid. The sites for aggregation might well be these β regions of the polypeptide. Although it has been suggested that the hydrophobic surfaces of α helices may be the sites of histone–histone interaction (Boublik et al., 1970), a survey of the literature shows many more examples of β sheet–β sheet interactions, e.g., crystalline insulin hexamers (Blundell et al., 1972). Thus it should be stressed that β-sheet interactions form more stable assemblies than associated α helices. Furthermore, kinetic studies on f2a1 show that aggregation accompanies β-sheet formation but not α-helix formation (Wickett et al., 1972; Smerdon and Isenberg, 1974). This hypothesis for histone interactions is also supported by the data of Ziccardi and Schumaker (1972, 1973), and Bradbury and coworkers (Bradbury and Rattle, 1972; Boublik et al., 1970). The conformational predictive scheme of Chou and Fasman (1974) supports the β-sheet potential of the C-terminal end; the entire segment from residue 80 through residue 102 is predicted to have a β-sheet conformation (with $\langle P_\beta \rangle = 1.22$), except for a β turn at residues 92-95. Thus, there is considerable evidence that this might well play an important role in aggregation and histone association.

It is not yet known what structural changes are responsible for the differences in circular dichroic spectra when DNA is incorporated into chromatin or reconstituted into histone complexes. DNA can retain its normal B-form helical geometry and still display a highly distorted CD pattern; this is shown in the case of Ψ-condensed DNA for which a CD pattern characterized by a large, negative band at 270 nm (Jordan et al., 1972) is consistent with an X-ray analysis showing B-form DNA (Maniatis et al., 1974). The periodic folding of DNA in the Ψ form is similar to a liquid crystal array, and may be present also in films of DNA under certain conditions (Brunner and Maestre, 1974) and possibly in chromosomes (Lerman, 1974). It has been shown that liquid crystalline spacings can give rise to circular dichroic bands near the absorption wavelength of the specimen (Holzwarth and Holzwarth, 1973). A particular structure in chromatin may well be accompanied by periodic spacings which could be responsible for distorted CD spectra.

Although f1–DNA complexes display Ψ-type CD spectra, those of f2a1–DNA complexes are very different, resembling RNA or A-form DNA. However, this chiral pattern may be due to specific folding of the DNA, and need not entail any change in geometry of the DNA double helix. In any case, it has been shown herein that the intact f2a1 molecule is mandatory for circular dichroic change in DNA upon complexation, and that the N and C fragments are ineffectual.

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Magnetic Circular Dichroic Spectra of Cobalt(II) Substituted Metalloenzymes†

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ABSTRACT: The magnetic circular dichroic (MCD) spectra of cobalt(II) substituted metalloenzymes have been studied and compared to a series of four-, five-, and six-coordinate cobalt(II) model complexes previously examined (T. A. Kaden et al. (1974), *Inorg. Chem. 13, 2585*). The MCD spectra of cobalt substituted carboxypeptidase A, procarboxypeptidase A, and thermolysin are consistent with earlier deductions of tetrahedral coordination from absorption spectra and also with X-ray structure analysis. Inhibitors fail to alter their MCD spectra significantly. The MCD spectra of cobalt alkaline phosphatase and carbonic anhydrase are more complex and their pH dependence and alteration by inhibitors are discussed in terms of known cobalt(II) models.

The substitution of metal atoms at the active sites of metalloenzymes is one of the mildest and most selective procedures currently available for the chemical modification of enzymes. Generally, such substitutions do not measurably alter overall protein conformation or structure and often preserve catalytic function (Vallee and Wacker, 1970; Davies et al., 1968; Holmquist and Vallee, 1974). When such substitutions replace a colorless, diamagnetic atom, e.g., zinc, with one which is chromophoric and paramagnetic, e.g., Co(II), considerable experimental advantages can accrue in correlating coordination geometries at the active sites with functional properties of metalloenzymes. Thus, the absorption spectra of a number of Co(II) substituted zinc metalloenzymes are unusual when compared to those of Co(II) complex ions of known structure (Vallee and Williams, 1968). Such spectra permit deductions both regarding the detailed and overall characteristics of active site geometry and the function of metals in metalloenzymes. Both electronic and magnetic resonance spectra constitute an important resource to enlarge upon deductions from X-ray crystallographic structure analysis and to extend its potential to the study of conformation and structure of enzymes in solutions.

Among the spectral approaches now feasible, the potential of magnetic circular dichroism (MCD)[1] remains relatively unexplored in defining Co(II) coordination in metalloenzymes though it would likely reveal information not previously accessible by other spectroscopic techniques presently in common use. Thus, while molar absorptivity

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[1] Abbreviations used are: MCD, magnetic circular dichroism; Me_6tren, tris(2-dimethylaminoethyl)amine; POA, *N*-2-picolyloxamide; Et_4dien, *N,N,N',N'*-tetraethyldiethylenetriamine; terpy, 2,2';6'2''-terpyridine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.