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Spectropolarimetric Analysis of the Core Histone Octamer and Its Subunits†

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ABSTRACT: The secondary structure of the calf thymus core histone octamer, $(H2A–H2B–H3–H4)_2$, and its two physiological subunits, the H2A–H2B dimer and $(H3–H4)_2$ tetramer, was analyzed by ORD spectropolarimetry as a function of temperature and solvent ionic strength within the ranges of these experimental parameters where assembly of the core histone octamer exhibits pronounced sensitivity. While the secondary structure of the dimer is relatively stable from 0.1 to 2.0 M NaCl, the secondary structure of the tetramer exhibits complex changes over this range of NaCl concentrations. Both complexes exhibit only modest responses to temperature changes. ORD spectra of very high and very low concentrations of stoichiometric mixtures of the core histones revealed no evidence of changes in the ordered structure of the histones as a result of the octamer assembly process at NaCl concentrations above 0.67 M, nor were time-dependent changes detected in the secondary structure of tetramer dissolved in low ionic strength solvent. The secondary structure of the chicken erythrocyte octamer dissolved in high concentrations of ammonium sulfate, including those of our crystallization conditions, was found to be essentially unchanged from that in 2 M NaCl when examined by both ORD and CD spectropolarimetry. The two well-defined cleaved products of the H2A–H2B dimer, cH2A–H2B and cH2A–cH2B, exhibited reduced amounts of ordered structure; in the case of the doubly cleaved moiety cH2A–cH2B, the reductions were so pronounced as to suggest marked structural rearrangements.

Because of their central role in the compaction and organization of eukaryotic chromosomes, the inner or core histones (H2A, H2B, H3, and H4) have been the objects of structural studies for many years. [For a review of histone and nucleosome structure, see McGhee and Felsenfeld (1980).] The early work of Isenberg, Kelly, and others established that histones H2A and H2B exist as a stable dimeric complex over a broad range of solvent conditions; likewise, histones H3 and H4 have been shown to form the stable tetrameric complex $(H3–H4)_2$ (D'Anna & Isenberg, 1974a,b; Kornberg & Thomas, 1974; Rubin & Moudrianakis, 1975; Roark et al., 1974, 1976; Moss et al., 1976a,b). Although it has been recognized for over 15 years that two each of the inner histones comprise the octameric core of the nucleosomes (Kornberg, 1974; Noll, 1974; Olins et al., 1976), several years elapsed before evidence was presented demonstrating octamer assembly from the stable dimer and tetramer complexes (Eickbush & Moudrianakis, 1978; Ruiz-Carillo & Jorcano, 1979; Jackson & Chalkley, 1981; Worcel et al., 1978; Earnshaw et al., 1982).

From sedimentation equilibrium studies, Godfrey et al. (1980) have shown that core histone octamer assembly in 2 M NaCl can be described by a two-step reversible process involving the two subunits and a hexamer intermediate:

$$H2A–H2B + (H3–H4)_2 \rightleftharpoons H2A–H2B–(H3–H4)_2$$

$$H2A–H2B + H2A–H2B–(H3–H4)_2 \rightleftharpoons (H2A–H2B–H3–H4)_2$$

Eickbush and Moudrianakis (1978) explored the temperature and solvent ionic strength dependencies of the core histone assembly system by gel filtration. These studies revealed that the subunit interactions which are strong in 2 and 4 M NaCl decrease markedly at lower salt concentrations. The temperature sensitivity of the system was also found to be unusually pronounced.

The question arises as to whether changes, if any, which can be detected in the secondary structure of the core octamer subunits as a function of temperature and solvent ionic strength correlate well with the sensitivity of the octamer assembly process to these experimental parameters. It is also of interest to know whether the assembly process itself is accompanied by any alteration in the secondary structure of the histones. A number of spectropolarimetric studies on the secondary structure of histones have been reported since the early work of Isenberg and his co-workers (D'Anna & Isenberg, 1974a–c; Moss et al., 1976a,b; Beaudette et al., 1981; Prevelige & Fasman, 1987; Park & Fasman, 1987; and references cited therein). These studies have examined histones under a variety of solvent conditions; however, none have attempted a *systematic survey* of the core histone octamer or its physiological subunits over the temperature and solvent ionic strength ranges

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within which subunit interactions are particularly sensitive.

We report herein on optical rotatory dispersion (ORD)[1] spectroscopic analyses of the core histone octamer, dimer, and tetramer conducted over temperature and solvent ionic strength ranges where the octamer assembly process exhibits pronounced sensitivity. In addition to these native species, two well-defined proteolytic cleavage products of the dimer, cH2A–H2B and cH2A–cH2B, were examined. These smaller moieties, which were prepared by controlled digestion of native dimer by the H2A–H2B-specific protease isolated in this laboratory (Eickbush et al., 1976), show lower interaction potentials with the native tetramer than does the native dimer (Eickbush et al., 1988). Finally, chick erythrocyte octamer was examined in high concentrations of ammonium sulfate by both CD and ORD to detect possible effects of these solvent environments on the secondary structure of the histones. This study is of particular interest since octamer crystals were isolated from high concentrations of ammonium sulfate and subsequently characterized by X-ray crystallography in this laboratory (Burlingame et al., 1985).

Materials and Methods

Several aspects of the experimental procedures are described in more detail than usual, because the history of handling of a histone preparation as well as the ionic environment in which it is studied can have adverse effects on the measurable optical parameters under investigation. These issues are discussed in more detail in the following paper (Baxevanis et al., 1990).

Reagent-grade chemicals and deionized water were used in all preparative procedures and experiments.

Preparation of Histones. Calf thymus and chicken erythrocyte histones were isolated by a modification of the salt extraction method previously used in this laboratory (Eickbush & Moudrianakis, 1978). Dimers and tetramers were isolated efficiently on a CM-cellulose (Whatman CM-52) column equilibrated with 0.1 M potassium phosphate and 1 mM EDTA, pH 7.5, and eluted with a 0.1–0.5 M linear KCl gradient; alternatively, fractionation was effected on a Pharmacia Fast Flow S preparative cation-exchange column as described previously (Elia & Moudrianakis, 1988). All isolation steps were performed at 4 °C, and with repeated additions of PMSF.

Calf thymus "core complex" histones were prepared by the method of Eickbush and Moudrianakis [1978; see also Godfrey et al. (1980)].

Singly and Doubly Cleaved H2A–H2B. Dimers were exposed to the H2A-specific protease characterized in this laboratory under two sets of digestion conditions (Eickbush et al., 1976; Elia & Moudrianakis, 1988). The first promotes selective cleavage of a pentadecapeptide from the C-terminus of H2A to form cH2A–cH2B. The second promotes, in addition to the above cleavage, a much slower removal of approximately 25 residues from the N-terminus of H2B to form cH2A–cH2B.

Protein Determinations. Protein concentrations were routinely estimated from optical densities: concn = $(OD_{277nm} - 2OD_{329nm})/A_{1mg/mL}$ assuming a fourth-power wavelength dependency for the scattering correction. Mass extinction coefficients for the calf thymus octamer and its subunits were calculated from the optical densities and the known mass concentrations of carefully prepared solutions of the proteins dissolved in an appropriate reference solvent. The mass concentrations of these solutions were calculated from total nitrogen determinations (Galbraith Laboratories, Inc., Knoxville, TN) and the published amino acid sequences of the calf thymus core histones (Isenberg, 1979). Extinction coefficients in other solvent systems were determined by standard gravimetric dilution procedures (Table I). Extinction coefficients for the "core complex" histones were calculated from the subunit values assuming absorption additivity. The mass extinction coefficient of the chicken erythrocyte octamer was verified to be identical with that of calf thymus core octamer by interferometric measurements made on solutions of the two proteins in an interferometer of the author's (J.E.G.) design; it was assumed that the two proteins possess identical refractive index increments. Chicken erythrocyte octamer exhibits only limited solubility in ammonium sulfate buffer systems above 2.0 M. In order to establish the extinction coefficients for the octamer dissolved in buffers containing ammonium sulfate in the 2.0–2.6 M range, the extinction coefficient of the octamer dissolved in 1.5 M ammonium sulfate (in which the proteins are highly soluble) was first established. Aliquots of octamer dissolved in this solvent system were dialyzed against higher concentrations of ammonium sulfate. The dialyzed samples were then centrifuged to remove any precipitated protein and their optical densities recorded. The solutions were then gravimetrically diluted back down to 1.5 M ammonium sulfate and their optical densities again read. Octamer concentrations in the original, higher molarity ammonium sulfate solvents were calculated from the dilution factors and the established extinction coefficient for the protein in 1.5 M ammonium sulfate.

Mass extinction coefficients for cH2A–H2B and cH2A–cH2B were calculated by assuming that the average extinction per tyrosine residue (Isenberg, 1979) is the same in the native and cleaved moieties. In light of the structural rearrangements which accompany the controlled cleavages leading to these species (see Results), the assumed values may deviate somewhat from the actual numbers; however, we do not feel that the error introduced by any discrepancies in the extinction coefficient values would significantly change the observed

Table I: A_{277} Values for the Histone Octamer and Its Subunits in Several Solvent Systems

	A_{277}[a]		
solvent system	H2A–H2B	$(H3–H4)_2$	octamer
0.4 M NaCl, 50 mM potassium phosphate, pH 7.5[b]	0.475	0.422	0.449
0.1 M potassium phosphate, 0.2 M KCl, pH 7.0[c]	0.477	0.425	0.451
0.2 M NaCl, 20 mM potassium phosphate, pH 7.5	0.475	0.417	0.446
1.1 M NaCl, 20 mM potassium phosphate, pH 7.5	0.477	0.427	0.452
2.0 M NaCl, 20 mM potassium phosphate, pH 7.5	0.484	0.444	0.464
1.5 M $(NH_4)_2SO_4$, 10 mM $Na_4P_2O_7$, pH 6.8			0.461
2.0 M $(NH_4)_2SO_4$, 10 mM $Na_4P_2O_7$, pH 6.8			0.457
2.3 M $(NH_4)_2SO_4$, 10 mM $Na_4P_2O_7$, pH 6.8			0.455
2.6 M $(NH_4)_2SO_4$, 10 mM $Na_4P_2O_7$, pH 6.8			0.454

[a] In OD units cm^{-1} mg^{-1} mL. [b] Nitrogen analysis solvent (see Materials and Methods). [c] CM-cellulose column elution buffer (see Materials and Methods).

[1] Abbreviations: CD, circular dichroism; ORD, optical rotatory dispersion; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; CM-cellulose, (carboxymethyl)cellulose; ^1H NMR, proton magnetic resonance; cH2A, cleaved histone H2A; cH2B, cleaved histone H2B.

trends. All optical density measurements were made in a Gilford Model 252 spectrophotometer using 1-cm path-length quartz cells.

Gel Electrophoresis. The purity of the isolated histones was assessed by electrophoresis on 20% acrylamide–0.1% NaDodSO$_4$ gels (Laemmli, 1970). Proteins were visualized by bathing the gels in 40% ethanol, 5% acetic acid, and 0.1% Coomassie Brilliant Blue R and subsequently destaining with 20% ethanol and 5% acetic acid. Gels of protein preparations used in this study revealed only the appropriate core histones with no detectable breakdown products.

Optical Rotatory Dispersion. ORD spectra were recorded in a Cary 60 spectropolarimeter. The instrument was calibrated with a sample of oxygenated human hemoglobin A (a gift of Dr. G. Ackers); the values for the extinction coefficient and the ORD spectrum in the peptide bond absorption region were from Hanisch et al. (1969). Some ORD spectra were recorded in a second Cary 60 for comparison purposes (see Results). Jacketed 0.1- and 0.5-cm path-length cells (Hellma Scientific) were used in all experiments, and the temperature was maintained to within 0.4 °C with an Aminco circulating water bath. Rotatory strengths were recorded at 10 preselected wavelengths in the peptide bond Cotton region (221–243 nm, inclusive) by manually setting the instrument at each wavelength and recording the rotatory strength for 30 s or more. This protocol significantly improved the precision of the measurements since it facilitated the calculation of average rotatory values at lower wavelengths where pen noise was encountered. Rotatory strengths are expressed in degrees centimeter squared per decimole of amino acid residue assuming a residue weight of 111 daltons.

For the ORD studies, unless otherwise indicated, proteins were dissolved in 10 mM HEPES and 0.5 mM EDTA, pH 7.4 (the "ORD solvent"), plus NaCl at concentrations ranging from 0.065 to 2.0 M. For the temperature and ionic strength dependency studies (Table II), protein concentrations ranged from 0.2 to 0.5 mg/mL. ORD values of the "core complex" histones and mixtures of the isolated subunits (Table III) were recorded at very high and very low protein concentrations, as indicated. The longer path-length cells (0.5 cm) were used in all spectral recordings of solutions with protein concentrations below 1.0 mg/mL. Protein solutions were clarified by ultracentrifugation (30 min at 30000 rpm in a Beckman 50TI rotor) before optical densities and ORD spectra were recorded.

Circular Dichroism. Circular dichroism measurements were made in a Jasco J-500C spectropolarimeter as described in the following paper (Baxevanis et al., 1990). Cells with a path length of 0.05 or 0.1 cm were used for this study.

Estimation of Secondary Structure. The ORD data were analyzed for the relative contributions of α-helix, β-sheet, and random coil to the secondary structures of the proteins by the method of Chen et al. (1974). The method treats the average length of the α-helices (expressed as number of residues) as an adjustable parameter since the rotatory strength of the α-helix is length dependent. However, because changes in secondary structure as a function of environmental conditions were the principal focus of this study, the average length (n) was arbitrarily fixed at 11.3, the average length of the helix segments present in the five proteins used as references by Chen et al. (1974). Best fits of model spectra were obtained by using nonlinear least-squares fitting programs written by the authors; these programs generate standard deviations from the residuals as a measure of closeness of fit [cf. Yang et al. (1986) and Adler et al. (1973) for a discussion of different methods used to fit CD and ORD spectra and a comparison of their accuracy in predicting secondary structure].

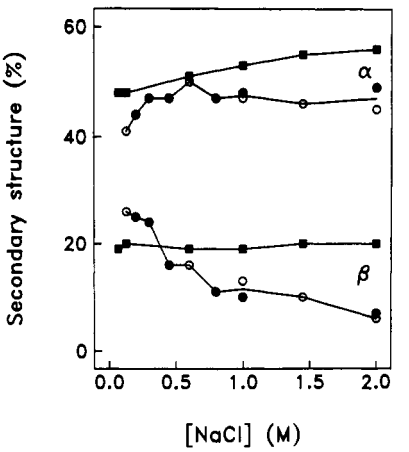

FIGURE 1: Estimates of secondary structure in dimers (■) and tetramers (●, ○) as a function of solvent ionic strength (M NaCl). (○) Samples initially incubated for 2 days in 1.0 M salt solvent (see Results); (●) samples initially incubated in 0.13 M salt solvent (see Results). Estimates are given as percent of total residues in the indicated (α or β) conformation. All spectra were recorded at 20 °C. Solvents also contained 10 mM HEPES and 0.5 mM EDTA, pH 7.4.

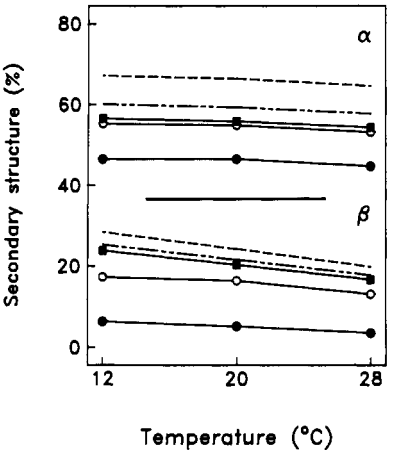

FIGURE 2: Estimates of secondary structure in native dimers (■), cH2A–H2B (○), and cH2A–cH2B (●) as a function of temperature. Estimates are given as percent of total residues in the indicated (α or β) conformation. Solvent was 2.0 M NaCl, 10 mM HEPES, and 0.5 mM EDTA, pH 7.4. (----) Secondary structure estimates expected for cH2A–H2B if cleaved pentadecapeptide is random coil in the native dimer; (--) secondary structure estimates expected for cH2A–cH2B if cleaved pentadecapeptide and cleaved 25-mer are both random coil in the native dimer (see text for details).

Results

Optical Rotatory Dispersion Studies. (a) H2A–H2B Dimer. The α-helical content of the native dimer decreased continuously with solvent ionic strength, dropping from 56% to 48% of the total residues (a loss of 20 residues) between 2.0 and 0.065 M NaCl (Table II; Figure 1). The β-sheet content remained constant at 20% over this salt range. Between 12 and 28 °C, the α-helical content of dimer dissolved in 2 M NaCl remained unchanged at 56%; however, approximately 17 residues of β-sheet were lost over the temperature range (Table II; Figure 2).

(b) $(H3–H4)_2$ Tetramer. The response of tetramers to changes in solvent ionic strength was more complex than that of the dimer. At 20 °C, the α-helical content remained constant between 2.0 and 0.5 M NaCl; however, below 0.5 M NaCl, it dropped from 47% to 41%, corresponding to a loss of about 28 residues. β-Sheet changes were more dramatic:

Table II: Estimates of Secondary Structure in Calf Thymus Core Histone Complexes from Analysis of ORD Spectra: Effects of Temperature and Solvent Ionic Strength

histone complex	solvent[a] (M NaCl)	temp (°C)	$[m']_{233}$[b]	$[m']_{224}$[b]	secondary structure[c] % α	% β	$\pm[m']$[d]
$(H3-H4)_2$	0.13	20	6660	2300	41	26	233
	0.2	20	7120	2050	44	25	232
	0.3	20	7410	2080	47	24	311
	0.45	20	7370	1480	47	16	184
	0.6	20	7670	1400	50	16	201
	0.8	20	7500	1100	47	11	122
	1.0	20	7230	987	48	10	135
	1.0	20	7250	1230	47	13	131
	1.45	20	7090	988	46	10	139
	2.0	20	7340	751	49	7	157
	2.0	20	6920	689	45	6	199
	2.0	12	6980	805	46	7	199
	2.0	28	6860	764	45	6	212
H2A-H2B	0.07	20	7470	1600	48	19	108
	0.13	20	7470	1670	48	20	75
	0.6	20	7860	1460	51	19	99
	1.0	20	8060	1450	53	19	93
	1.45	20	8260	1400	55	20	106
	2.0	20	8440	1370	56	20	69
	2.0	12	8600	1560	57	24	70
	2.0	28	8220	1150	54	17	86
cH2A-H2B	0.13	20	7570	1360	50	15	127
	0.2	20	7490	1280	49	14	139
	0.6	20	7800	1250	51	16	128
	1.3	20	8180	1190	54	16	117
	2.0	20	8250	1110	55	16	82
	2.0	12	8340	1200	55	17	87
	2.0	28	8040	990	53	13	146
cH2A-cH2B	2.0	20	7064	653	46	5	325
	2.0	12	7094	704	46	6	344
	2.0	28	6765	654	45	4	403

[a] Solvent also contained 10 mM HEPES and 0.5 mM EDTA, pH 7.4. [b] Experimental rotatory strengths are listed at the wavelengths where α-helix (233 nm) and β-sheet (224 nm) rotatory strengths are maximal (Chen et al., 1974). Units are degrees centimeter squared per decimole of amino acid residue. [c] Secondary structure estimates are percent of total residues in the indicated conformation; based on reference spectra for α-helix, β-sheet, and random coil derived from ORD spectra of five globular proteins of known structure (Chen et al., 1974). [Cf. Yang et al. (1986) and Adler et al. (1976) for a discussion of different methods used to fit CD and ORD spectra and a comparison of their accuracy in predicting secondary structure.] [d] Standard deviations based on residuals between experimental and fitted spectra at 10 wavelengths between 221 and 248 nm, inclusive. Units are degrees centimeter squared per decimole of amino acid residue.

from 2.0 to 0.13 M NaCl, tetramers gained 96 residues of β-structure, increasing from 6% to 26% of the total secondary structure.

The salt dependency study was carried out on two sets of tetramer samples which differed in their preparative protocols. This was done in order to test for two possible complications: (1) aggregation of tetramers in high ionic strength solvents (Rubin & Moudrianakis, 1975; unpublished observations); and (2) time-dependent changes in tetramer secondary structure in low ionic strength solvents, evidence for which has been reported by several workers (Lewis, 1976; Feldman et al., 1980). One set of samples (open circles, Figure 1) was prepared from a high concentration tetramer stock solution which had been dialyzed for 2 days against 1.0 M NaCl solvent; the different salt concentrations required for the ORD recordings were produced by the addition of the ORD solvent containing either no NaCl or 5 M NaCl. The second set of samples (closed circles, Figure 1) were prepared in similar fashion from a stock solution of tetramer which had been dialyzed for 2 days against 0.13 M NaCl solvent. Within 4 h of the last preparation step, samples were clarified by ultracentrifugation, their concentrations determined, and their ORD spectra recorded. The two sets of points appear to describe the same response to changes in bulk solvent ionic strength.

The concomitant gain of β-structure and loss of α-helix by tetramer when it is transferred from a high-salt to a low-salt environment can be clearly seen when the ORD spectra recorded under the two solvent conditions are superimposed

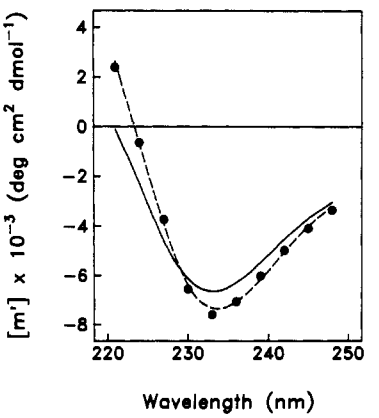

FIGURE 3: ORD spectra of tetramers in the negative Cotton trough region of the peptide bond absorption band at two solvent ionic strengths: (—) in 0.13 M NaCl; (- - -) in 2.0 M NaCl (see Table II for details). Points are the fitted spectrum to the experimental spectrum of tetramer in 2.0 M NaCl; standard deviation = 157 deg·cm²/dmol of amino acid residue.

(Figure 3). The negative Cotton trough due to α-helix conformation has its minimum (maximum rotatory strength) at approximately 233 nm; the β-structure minimum occurs at 224 nm, where the α-helical rotatory strength is close to zero (Chen et al., 1974). As a result of the structural changes, the spectra recorded under the two solvent conditions cross in the wavelength region between the two minima.

Tetramer, unlike dimer, was quite stable over the modest

Table III: Estimates of Secondary Structure in Calf Thymus Core Histone Complexes from Analysis of ORD Spectra: Effect of Subunit Interaction

expt	histone complex	solvent[a] (M NaCl)	concn (mg/mL)	(H + O)[b] (%)	$[m']_{233}$[c]	$[m']_{224}$[c]	secondary structure[d] % α	% β	±[m'][e]
1	H2A–H2B	2.0	2.2		8180	890	54	13	104
	$(H3–H4)_2$		1.9		6940	940	45	9	187
	H2A–H2B + $(H3–H_4)_2$		2.0	91	7780	860	51 (50)	11 (11)	111
2	H2A–H2B	1.0	1.5		8510	1470	56	22	117
	$(H3–H4)_2$		1.5		6630	980	43	8	195
	H2A–H2B + $(H3–H4)_2$		1.5	42	7560	1290	49 (50)	15 (15)	123
3	H2A–H2B + $(H3–H4)_2$[f]	0.67[g]	5.3	~0	7920	1290	52	17	114
			0.11	~0	7880	1490	51	19	158
4	H2A–H2B + $(H3–H4)_2$[f]	1.0[g]	4.8	55	8030	1160	53	16	99
			0.10	17	7690	1360	50	17	130
5	H2A–H2B + $(H3–H4)_2$[f]	2.0[g]	1.7	90	7540	840	50	10	185
			0.08	44	7480	900	49	10	195

[a] Solvent also contained 10 mM HEPES and 0.5 mM EDTA, pH 7.4. All spectra were recorded at 22 °C. [b] Percent, by weight, of hexamer and octamer present in mixtures of the subunits; based on assembly parameters in Godfrey et al. (1980) and unpublished results. [c] Experimental rotatory strengths are listed at the wavelengths where α-helix (233 nm) and β-sheet (224 nm) rotatory strengths are maximal (Chen et al., 1974). Units are degrees centimeter squared per decimole of amino acid residue. [d] Secondary structure estimates are percent of total residues in the indicated conformation; based on reference spectra for α-helix, β-sheet, and random-coil conformations derived from ORD spectra of five globular proteins of known structure (Chen et al., 1974). Numbers in parentheses are the percent of the indicated structure assuming additivity of the spectra of the individual subunits. [Cf. Yang et al. (1986) and Adler et al. (1976) for a discussion of different methods used to fit CD and ORD spectra and a comparison of their accuracy in predicting secondary structure.] [e] Standard deviation based on residuals between experimental and fitted spectra at 10 wavelengths between 221 and 248 nm, inclusive. Units are degrees centimeter squared per decimole of amino acid residue. [f] "Core complex" histones; molar ratio of dimers to tetramers = 0.67 (Godfrey et al., 1980). [g] Solvent contained 10 mM Tris in place of 10 mM HEPES.

temperature range examined. The α-helical content of tetramer in 2.0 M NaCl solvent remained unchanged at 45% between 12 and 28 °C; β-sheet content decreased slightly, from 7% to 5%, over the same range.

(*c*) *Singly and Doubly Cleaved Dimer.* The responses of cH2A–H2B to changes in temperature and ionic strength were similar to those of the native dimer (Table II; Figure 2). However, the absolute amounts of both α-helix and β-sheet were reduced in the cleaved product which lacks 15 residues from the C-terminus of the H2A moiety. Evidence from ^1H NMR spectroscopy and primary structure conformation analysis (Moss et al., 1976a) suggests an unordered conformation for the 14 C-terminal residues of calf thymus H2A. The loss of 14 residues of random coil from the dimer should reduce the fraction of unordered structure and thereby increase the fractions of α-helix and β-sheet in the degraded product (Figure 2). The experimental finding of a lower fraction of ordered residues in cH2A–H2B than in the native protein strongly suggests that structural modifications take place in the cleaved product as a result of the proteolysis.

Further selective degradation of the dimer—the removal of approximately 25 residues from the N-terminus of H2B (Elia & Moudrianakis, 1988)—results in additional, more pronounced reductions of ordered structure in the remaining moiety (Table II; Figure 2). In 2 M NaCl at 20 °C, cH2A–cH2B was found to contain approximately 21 fewer residues folded into α-helix than are present in cH2A–H2B (a decrease of from 55% to 46% α-helix). More strikingly, 28 of the 40 residues of β-sheet present in the singly cleaved product are no longer contributing to the β-sheet content of the doubly cleaved product; they are presumed to have taken an unordered conformation. Although no experimental evidence bears on the conformation of the 25-mer removed from H2B, primary sequence conformation analysis suggests that this region lacks any ordered structure (Moss et al., 1976a). Figure 2 shows the expected α- and β-structure contents of cH2A–cH2B assuming a random coil conformation for all 25 residues removed; however, even if some ordered structure is present in this peptide in the native complex, it is clear that major losses of both α-helix and β-sheet in the remaining core moiety accompany its removal.

(*d*) *Mixtures of Dimer and Tetramer.* The ORD spectra of dimer–tetramer mixtures in NaCl environments at or above 0.67 M were recorded and compared with the spectra of the individual isolated subunits to determine the effect of histone assembly on secondary structure. In one set of experiments, solutions of the isolated subunits were combined at a dimer to tetramer molar ratio of 2:1 (the ratio present in the core octamer), and the ORD spectrum of the resulting mixture was recorded and compared with the ORD spectra of the isolated subunits (experiments 1 and 2, Table III). In both 1.0 and 2.0 M NaCl, the ORD spectra of the mixtures closely approximated the composite spectra generated by averaging the spectra of the isolated subunits; the contributions from the subunits were thus additive to within the experimental error of the spectral recording procedure.

In the second set of experiments, the ORD spectra of "core complex" histones [that is, the H2A–H2B dimer and $(H3–H4)_2$ tetramer] were examined in 0.67, 1.0, and 2.0 M salt solvents (experiments 3–5, Table III). Two protein samples were measured at each salt concentration: one at a high protein concentration and the other at a very low protein concentration; the second protein sample was a quantitative dilution of the first. Both a high and a low protein concentration sample was measured since the assembly of the individual dimer and tetramer subunits is dependent on protein concentration as well as ionic strength (column 4, Table III; Godfrey et al., 1980). The spectra of the low and high protein concentration sample of "core complex" histones in 0.67 M NaCl were indistinguishable; this is not unusual, as minimal subunit interactions occur in solvents at this ionic strength (Eickbush & Moudrianakis, 1978). More significantly, the spectra from histones dissolved in 1.0 and 2.0 M NaCl were also identical. In other words, there is no significant net change in secondary structure as the ionic strength of a histone solution is increased nor as the histone concentration itself is increased; i.e., the secondary structure of the core histones is not sensitive to assembly.

(*e*) *Core Octamer in High Concentrations of Ammonium Sulfate.* ORD spectra of this phase of the study were recorded in a second Cary 60 spectropolarimeter (courtesy of Dr. W. F. Harrington). The ORD spectra of chicken erythrocyte

Table IV: Estimates of Secondary Structure of Calf Thymus and Chick Erythrocyte Core Histones from ORD Spectra: Effects of High Concentrations of Ammonium Sulfate

tissue	solvent[a]	$[m']_{233}$	$[m']_{224}$	% α	% β	$\pm[m']$
calf thymus	2.0 M NaCl	7729	362	52	5	180
calf thymus	2.6 M $(NH_4)_2SO_4$	8026	353	54	5	165
chick erythrocyte	2.0 M NaCl	7729	694	53	6	237
chick erythrocyte	2.3 M $(NH_4)_2SO_4$	8068	533	54	8	174
chick erythrocyte	2.3 M $(NH_4)_2SO_4$, +0.2 nm[b]	8102	799	54	12	136
chick erythrocyte	2.3 M $(NH_4)_2SO_4$, −0.2 nm[b]	7979	244	54	4	205
chick erythrocyte	2.6 M $(NH_4)_2SO_4$	8059	466	54	7	167

[a] Ammonium sulfate solvents also contained 10 mM sodium pyrophosphate and 0.25 mM EDTA, pH 6.8; all spectra were recorded at 22 °C. [b] Rotatory strengths read at each of the 10 reference wavelengths offset by either plus (+) or minus (−) 0.2 nm. See legend to Table III for additional details.

octamer dissolved in ORD solvent plus 2 M NaCl were compared with those of the octamer dissolved in 2.3 and 2.6 M ammonium sulfate, all solutions also containing 10 mM sodium pyrophosphate and 0.25 mM EDTA, pH 6.8. It can be seen that no significant changes in the amounts of either α- or β-structure occurred in the octamer as a result of transferring the protein to the higher ionic strength ammonium sulfate systems (Table IV). Calf thymus octamer dissolved in the 2 M NaCl and 2.6 M ammonium sulfate buffers had similar spectra and secondary structure profiles. Similarly, chicken erythrocyte core histones dissolved either in 2 M NaCl, 10 mM HEPES, and 1 mM EDTA, pH 7.5, or in 2.6 M ammonium sulfate, 10 mM sodium pyrophosphate, and 0.25 mM EDTA, pH 7.2, were examined by CD spectroscopy. No significant net changes in secondary structure are seen for core histones in these two solvents: Core histones in the 2 M NaCl solvent had 55% α-helix and 4% β-structure, with $[\theta]_{222} = -15\,200 \pm 100$ deg cm^2/dmol; core histones in the 2.6 M ammonium sulfate solvent also had 55% α-helix and 4% β-structure, with $[\theta]_{222} = -14\,800 \pm 300$ deg cm^2/dmol.

The spectra of the two octamers dissolved in the 2 M NaCl solvent system recorded in the second Cary 60 gave lower estimates of β-structure than those obtained by using the Cary 60 used in the first part of the study (Table III). This discrepancy prompted us to determine the wavelength sensitivity of the secondary analysis which, if present, could point to a slight mismatch between the wavelength scales of the two instruments. In other words, we performed an experiment in which we deliberately offset one of the spectropolarimeters by the minimum discernible increment (0.2 nm) to find whether such a small error had a significant effect on the signal obtained. The ORD spectrum of the chick erythrocyte octamer sample dissolved in 2.3 M ammonium sulfate was recorded at each of the 10 wavelengths at which rotatory strengths were obtained with the wavelength offset by ±0.2 nm. Structural analyses of the two resulting spectra gave dramatically different apparent amounts of β (but not α) structure (Table IV). These results suggest that the discrepancies between the amounts of β-structure obtained from the spectra of identical solutions of core octamer recorded in the two instruments were indeed due to a slight mismatching (e.g., 1–2 Å) of the wavelength scales which, unless specifically monitored for, can go unnoticed.

Discussion

The secondary structure of solvated histones has been the focus of several other studies in recent years [see Beaudette et al. (1981) and references cited therein], but in every instance, these studies employed circular dichroism rather than optical rotatory dispersion as the spectropolarimetric technique of choice. Our use of the older spectropolarimetric method for the present study was based on two considerations which we felt overrode the principal advantage of CD[2] [see Sears and Beychok (1973) and Imahori and Nicola (1973) for discussions of the relative merits of the two techniques]. First, the α-helix and β-sheet negative Cotton troughs recorded by ORD are displaced up-wavelength to a significant degree from their corresponding CD absorption bands. Not only does protein absorption increase dramatically below 220 nm due to the peptide bond, but solvent constituents, notably EDTA, strongly absorb in this region as well. Thus, the ORD spectra of the present study were recorded under more favorable conditions of the signal-to-absorption ratio than would have been the case with the corresponding CD spectra yielding the same data.

Second, the α-helix and β-sheet Cotton troughs are more comparable in size and overlap to a far lesser extent than the CD spectra of these structures. Indeed, the β-structure CD spectrum is completely encompassed by the much larger α-helix CD spectrum, whereas the maximum rotatory strength of the β-sheet Cotton trough occurs at a wavelength (224 nm) where the α-helix rotatory strength is close to zero (Chen et al., 1974). Therefore, we believe that ORD can often yield a more sensitive measure of changes in β-structure within a molecule in response to altered experimental parameters. However, we do recognize that because of the dispersed signal generated by ORD, comparisons of the secondary structures of different molecules may be less reliable by ORD than by CD (Imahori & Nicola, 1973). In addition, as the wavelength dependency study reported herein demonstrates (Table IV), accurate estimates of β-structure can only be obtained from spectra reflecting precise wavelength scale alignment.

Very good fits were obtained between the experimental spectra and the corresponding model spectra. A typical example of the fit between an experimental ORD spectrum and its corresponding model spectrum fitted to it is seen in Figure 3. In general, it is reasonable to assume that the better the fit, the more reliable are the estimates of changes in the secondary structure of a protein accompanying changes in experimental parameters. Estimates of absolute structural contents are probably less reliable due principally to the dependence of the α-helix rotatory power on the length of the helical segment and also the dependence of the rotatory power of the randomly disposed amino acid sequences on the primary structure of the protein (Chen et al., 1974; Imahori & Nicola, 1973).

Secondary structural changes do not appear to accompany

[2] The primary advantage of the CD technique lies in the fact that an optically active chromophore yields a CD spectrum only in the wavelength region of its absorption band, whereas ORD exhibits an attenuated dispersion signal above and below the absorption (Cotton) region. Thus, CD spectra in an *absorption band of interest* are more apt to be free of contributions from active chromophores other than the one(s) which absorb(s) in that region.

native subunit associations in sodium chloride solvents in the ionic strength range where octamer assembly is favored (Table II). In 1.0 and 2.0 M salt, the ORD spectra of mixtures of dimers and tetramers yielded α- and β-structure contents very close to the values calculated from the spectra of the isolated subunits assuming simple additivity (values in parentheses in Table III). No changes were observed in the ordered structure of "core complex" histones dissolved in 1.0 and 2.0 M salt as a result of disassembly of associated species by the dilution procedure. These findings appear to be at variance with the observation reported by Beaudette et al. (1981) that "core complex" histones at high concentration which had been isolated by a high-salt extraction method have a CD spectrum which differs from that obtained with equimolar mixtures of the isolated subunits. Philip et al. (1980) have also reported a systematic decrease in the α-helix content of the "core complex" histones upon dilution. These apparent differences could be the result of specimen preparation procedures.

Of the two native subunits, the dimer was found to be the more stable within the temperature and ionic strength ranges examined. A small, continuous decrease in the α-helix content occurs with decreasing solvent ionic strength (Figure 1), and a mild temperature lability of the β-sheet content was observed (Figure 2). These results are in general qualitative agreement with those of Beaudette et al. (1981). However, from CD analyses, these authors found the β-sheet content in the dimer dissolved in neutral pH solvents at 4 °C to be only 3–6%, an amount substantially less than the 24% β-sheet we detected in dimer dissolved in 2.0 M NaCl at 12 °C.

The secondary structure of H3–H4 tetramer was quite stable to temperature changes; however, varying the solvent ionic strength elicited pronounced changes in the β-sheet content and lesser changes in α-helix (Figure 1). Lowering the salt concentration from 2.0 to 0.13 M increased the β-structure content by 96 residues (from 6% to 26% of the total structure). It should be noted, however, that approximately half of this increase appears to have occurred below 0.5 M salt. The α-helix content was essentially unchanged between 2.0 and 0.5 M salt but decreased moderately in the ionic strength range between 0.5 and 0.13 M (a loss of approximately 29 residues). Beaudette et al. (1981) reported a small (6%) increase in β-structure when the solvent salt was lowered from 2.0 M to approximately 0.02 M. These authors also reported a large loss of α-helical structure, approximately 87 residues (from 44% to 26%) with the same decrease in ionic strength. Taken together, the results from the two studies suggest that the major reduction in the β-structure content of tetramer takes place upon decreasing the solvent ionic strength below physiological values (approximately 0.13 M).

Because dimer–tetramer interactions are significantly reduced at NaCl concentrations below 0.5 M, the major alterations in ordered structure occurring in the tetramer and the less dramatic changes in the α-helical content occurring in the dimer throughout this low-salt range probably do not have a direct effect on the assembly of the octamer, nor can they be considered indicators of this assembly. However, these changes may be a prelude to assembly, reflecting rearrangements of the subunit surfaces that facilitate or enable subsequent assembly at higher salt concentrations. The more modest changes in β-structure which occur in the tetramer and the changes in β-structure which occur in the dimer over the 0.5–2.0 M NaCl concentration range where subunit interactions are favored may play a role in the salt concentration dependence of these interactions. Accordingly, these structural changes may alter the binding between the subunits, but as reported in the previous section, there is no evidence to indicate that assembly of the octamer is accompanied by significant alterations in the ordered structure of the subunits, i.e., that assembly imposes a specific secondary structure onto the dimer and/or tetramer.

The protocol followed in preparing samples for the ionic strength dependency study of tetramers was designed to test for the presence of slow changes in secondary structure content when tetramer is incubated in low-salt solvents, a conclusion reached earlier by at least two authors (Feldman et al., 1980; Lewis, 1976). Samples of tetramer in solvents representative of the salt concentration range examined by these authors were prepared from stock solutions which had been dialyzed for 2 days against ORD solvent containing either 1.0 or 0.13 M NaCl. Both sets of samples gave essentially the same results (Figure 1). Thus, no relatively slow, time-dependent changes (i.e., continuing beyond 3–4 h; see Results) prompted by either increasing or decreasing the solvent ionic strength were detected in the tetramer ORD spectra of our tetramer preparations.

It should be noted that solutions of isolated tetramer aggregate over time (as evidenced by the increase in the apparent absorption at 329 nm; see Results) and the rate of aggregation seems to be faster in high-salt solvents. It is well established that aggregation in protein solutions can cause distortions in ORD and CD spectra in the peptide bond region (Urry & Krivacic, 1970). Therefore, we have found that it is critical that solutions of tetramer be clarified just prior to protein concentration determinations and experimental analysis. Protease contamination must also be avoided; the H3 moiety of tetramer is particularly susceptible to attack in low-salt buffers (Bartley & Chalkley, 1970; unpublished observations).

The problem of sample aggregation also arises when the core histones are dissolved in high concentrations of ammonium sulfate. Through monitoring changes in absorbance at 277 and 329 nm, we have found that there is an appreciable amount of octamer salting out in ammonium sulfate solvents above 2.0 M; indeed, we take advantage of this property of the octamer in our histone preparative procedure (see Materials and Methods). Thus, as with tetramer samples, samples of octamer dissolved in ammonium sulfate buffers require clarification before their concentrations are determined and their spectra recorded. Once clarified, however, these samples remain stable and turbidity-free for several hours.

Both cleaved products of native dimers, cH2A–H2B and cH2A–cH2B, contain lower amounts of ordered structure than the native parent complex. The removal of 15 residues from the C-terminus of the H2A moiety causes a small reduction in α-helix content and a somewhat larger loss of β-sheet. If, as evidence suggests (Moss et al., 1976a,b), the domain of the cleaved peptide is randomly ordered in the native complex, then our results require that the remaining moiety, cH2A–H2B, undergo a loss of approximately 25 residues of ordered structure (10% of the total number of residues) in response to the proteolysis (Figure 2). Evidence from ^{1}H NMR spectroscopy (Moss et al., 1976b; Bradbury et al., 1977) indicates that the pentadecapeptide is not involved in the tertiary structure of the H2A–H2B complex; given this interpretation of the ^{1}H NMR data, it would be all the more striking that removal of the peptide should prompt a disruption of the conformation of the remaining polypeptide moiety.

The removal of the second peptide from the dimer causes additional alterations of the ordered structure of the remaining cleaved product, cH2A–cH2B. The α-helical content is further reduced, and approximately three-quarters of the β-sheet

structure is unfolded. If the 25-mer domain of the amino terminus of H2B is randomly disposed as primary sequence analysis of this region suggests (Moss et al., 1976a,b; Bradbury et al., 1977), as many as 63 residues of ordered structure in the remaining moiety are required to be converted to random conformation when the peptide is cleaved from cH2A–H2B. The removal of one or both of the peptides from the native dimer reduces the octamer assembly potential of the remaining moiety (Eickbush et al., 1988); indeed, cH2A–cH2B has little affinity for tetramers in high-salt solvents.

We observed no changes in the secondary structure of either calf thymus or chick erythrocyte histone octamer when it is dialyzed from 2 M NaCl buffers into high concentrations of ammonium sulfate. We also found no changes in the secondary structure of the latter octamer within the 2.0–2.6 M ammonium sulfate concentration range. These findings lend support to the conclusion that the structure of the chicken erythrocyte octamer molecules within the crystals formed in ammonium sulfate solvent and characterized by X-ray crystallography in this laboratory (Burlingame et al., 1985) are representative of the structures of the solvated histone octamer widely studied in 2 M NaCl and other high-salt buffers. A more detailed analysis of the CD response of the core histone octamer in 2.0–2.5 M ammonium sulfate is presented in the following paper (Baxevanis et al., 1990).

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