Associative Behavior of the Histone $(H3–H4)_2$ Tetramer: Dependence on Ionic Environment†

Andreas D. Baxevanis, Jamie E. Godfrey, and Evangelos N. Moudrianakis*
Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218
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ABSTRACT: Mixtures of histones H3 and H4 were examined by analytical ultracentrifugation and circular dichroism to determine their association behavior and secondary structure content in high and low ionic strength solvents containing chloride, phosphate, or sulfate. H3 and H4 were also cross-linked by using DSP in order to directly trap any intermolecular interactions occurring in solution. While H3 and H4 can exist as an H3–H4 dimer under limited conditions, they behave as a stable $(H3–H4)_2$ tetramer under most conditions, particularly those which are physiologically relevant. In chloride-containing solutions, the equilibrium between H3–H4 and $(H3–H4)_2$ is responsive to changes in ionic strength and paralleled by large changes in α-helicity. In sulfate- and phosphate-containing solutions, the equilibrium is again governed by ionic strength, but there are no significant changes in secondary structure accompanying shifts in the equilibrium. Small oligomers can be formed in the presence of sulfate and phosphate and trapped by the cross-linking reagent; these oligomers are much smaller than those formed in chloride-containing solutions. However, addition of the H2A–H2B dimer into the system prevents aggregation of the $(H3–H4)_2$ tetramer by acting as a "molecular cap" and thus regulating the assembly pathway toward the formation of tripartite octamers. The observed assembly of H3 and H4 into a stable, tetrameric complex supports the concept of the core histone octamer having a tripartite organization in solution rather than being organized as two heterotypic tetramers.

It has been well established that the basic repeating unit of chromatin structure, the nucleosome, is composed of an octameric assembly of two each of the four core histones (H2A, H2B, H3, and H4), around which is wrapped approximately 146 base pairs of DNA. Since the first observations that the core histone proteins exist as an octamer (Kornberg & Thomas, 1974), there has been some debate over what the physiological subunits of the octamer are. One view holds that the octamer dissociates into symmetrical halves which both contain one of each of the four core histones (a "heterotypic tetramer", i.e., H2A–H2B–H3–H4). This model was developed to account for low-speed sedimentation, laser light scattering, and cross-linking data (Weintraub et al., 1975; Campbell & Cotter, 1976; Chung et al., 1978) obtained for the octamer in 2 M NaCl which seem to indicate that the molecular weight of the main histone species in these solutions is an assembly of 55 000; this value would correspond, among other things, to an assembly of one each of H2A, H2B, H3, and H4. In contrast, another view holds that the core histone octamer is comprised of a central $(H3–H4)_2$ tetramer flanked by two H2A–H2B dimers in an overall tripartite configuration. The tripartite model is strongly supported by gel filtration experiments (Eickbush & Moudrianakis, 1978) which showed that the octamer, stable at 2 M NaCl, pH 7.5, is in a reversible equilibrium with H2A–H2B dimers and $(H3–H4)_2$ tetramers, the equilibrium being responsive to changes in ionic strength, pH, urea concentration, or temperature. Furthermore, high-speed (meniscus depletion) sedimentation experiments (Godfrey et al., 1980), which allow the detection of protein species even at very low concentration, yielded data which support the binding of two H2A–H2B dimers to an $(H3–H4)_2$ tetramer to form the histone octamer. This model is in accord with the results of cross-linking experiments which indicate that there is no time-dependent buildup of cross-linked heterotypic tetramers as an intermediate before formation of a cross-linked octamer (Thomas & Kornberg, 1975; Thomas & Butler, 1977).

The central difference between these two views is the state of assembly of H3 and H4 upon the dissociation of the histone octamer, that is, whether the octamer is dissociating along the two H2A–H2B dimer–$(H3–H4)_2$ tetramer interfaces or along an intra-$(H3–H4)_2$ tetramer interface. In the first case, the resulting assemblies in solution will have molecular weights of 27 000 and 53 000, respectively; in the second case, a single assembly with a molecular weight of 55 000 will be observed. Results from an early sedimentation experiment performed on a mixture of H3 and H4 in a *low* ionic strength solution at *low* pH indicated that these two polypeptides exist primarily as an H3–H4 heterodimer and that this dimer can undergo a "concentration-dependent reversible association" to form an $(H3–H4)_2$ tetramer (Roark et al., 1974). In the present study, we systematically examine the isolated histone tetramer by analytical ultracentrifugation and circular dichroism to determine its molecular weight, association behavior, and secondary structure content in *low and high* ionic strength and in a variety of solvent systems. We have also cross-linked the tetramers at neutral pH in these solvents using dithiobis(succinimidyl propionate) (DSP)[1] (Lomant & Fairbanks, 1976), a homobifunctional *N*-hydroxysuccinimide ester, to directly trap the molecular assemblies which develop between

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* To whom correspondence should be addressed.

[1] Abbreviations: DSP, dithiobis(succinimidyl propionate) or Lomant's reagent; PMSF, phenylmethanesulfonyl fluoride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; CM-cellulose, (carboxymethyl)cellulose; EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism; TCA, trichloroacetic acid; Γ, ionic strength.

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H3 and H4 in solution. The data suggest that the ionic strength and specific chemical composition of solutions containing H3 and H4 can markedly influence the equilibrium between the H3–H4 dimer and the $(H3–H4)_2$ tetramer, but under most conditions, including those which are physiologically relevant, the predominant assembly is the $(H3–H4)_2$ tetramer. The relevance of this observation, in light of previously reported findings and, more importantly, the high-phosphate environment to which the tetramer is exposed when bound to DNA, is discussed.

Experimental Procedures

Histone Isolation. Chicken erythrocyte histones were isolated by a modification of the salt extraction procedure 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/1 mM EDTA, pH 7.5, and eluted by using a 0.2–1.0 M stepwise KCl gradient. All isolation steps were performed at 4 °C, with repeated additions of PMSF.

Circular Dichroism. Stock chicken erythrocyte tetramers were diluted approximately 20-fold into either 0.01–2 M NaCl, 0.01–0.5 M sodium phosphate, 0.01–0.5 M Na_2SO_4, 0.01–0.5 M $(NH_4)_2SO_4$, or 0.01–2 M NH_4Cl; the sulfate and chloride solutions also contained 10 mM HEPES, pH 7.5, and 1 mM EDTA, while the phosphate solutions were 1 mM EDTA at pH 7.5 (cf. Table I). Circular dichroism measurements were made in a Jasco J-500C spectropolarimeter (courtesy of Dr. Yong Shin, National Institutes of Health Gerontology Research Center, Baltimore, MD). All measurements were made at a working tetramer concentration of 0.2–0.3 mg mL^{-1} in a 0.1-cm path-length quartz cell (Hellma Scientific) at 20.0 ± 0.1 °C; the temperature of the sample compartment was regulated by a Lauda K-2/R refrigerated bath-circulator (Brinkmann). For each sample, ellipticities were determined by direct reading at 12 manually set wavelengths (207–240 nm at 3-nm intervals) after the signals had stabilized for at least 30 s, the rationale for this procedure having been explained earlier (Godfrey et al., 1990). Molar ellipticities were calculated in units of degrees centimeter squared per decimole, using 111.1 as the formula weight of an average amino acid residue. The data were analyzed for the relative contributions of α-helix and β-sheet to the secondary structure of the proteins by using the method of Chen et al. (1974) as applied by Godfrey et al. (1990) for use with the histone proteins. In the present study, the small residuals obtained from the nonlinear least-squares analysis indicate good agreement of the experimental spectra with the model spectra and that the observed changes in ellipticity are significant [cf. Yang et al. (1976) and Adler et al. (1973) for a discussion of different methods used to fit CD spectra and a comparison of their accuracy in predicting secondary structure].

CD spectra were attempted in NaOAc-, NaI-, $NaNO_2$-, $NaNO_3$-, and $NaHCO_3$-containing buffers, but spectra could not be obtained since the solvents themselves are strong absorbers of ultraviolet light. While sodium perchlorate is itself a satisfactory buffer system for obtaining CD measurements, changes in A_{329} of tetramers in relatively low concentrations of $NaClO_4$ indicate a time-dependent aggregation of the protein; this behavior is currently under investigation.

Chemical Cross-Linking. Tetramers were dialyzed into one of the following solvent systems: (1) 10, 100, 250, or 500 mM sodium phosphate, pH 7.5, each containing 1 mM EDTA; (2) 10 mM, 100 mM, 1 M, or 2 M NaCl, each containing 10 mM HEPES, pH 7.5, and 1 mM EDTA; or (3) 10, 100, 250, or 500 mM Na_2SO_4, each containing 10 mM HEPES, pH 7.5, and 1 mM EDTA. The samples were removed from dialysis and clarified by ultracentrifugation at 30000 rpm for 30 min in a Beckman TI50 rotor. The concentration of each tetramer solution was determined spectrophotometrically (Godfrey et al., 1990), after which solutions were adjusted to 0.75 mg/mL. Tetramers were subjected to chemical cross-linking by the addition of DSP (Pierce Chemical) to a final concentration of 0.5 mM. After 30 min at 0 °C, the cross-linking reaction was terminated by precipitating the proteins with 25% TCA and washing the precipitate with acetone as described by Thomas (1989). Products were analyzed by boiling the samples for 5 min and applying them to 15% acrylamide–0.1% $NaDodSO_4$ gels (Laemmli, 1970). The amount of protein applied to each of the lanes was adjusted so that the presence and position of all cross-linked products would be easily detectable. Proteins were visualized by bathing the gels in 40% ethanol, 5% acetic acid, 0.1% Coomassie Brilliant Blue R (Sigma Chemical) and subsequently destaining with 20% ethanol and 5% acetic acid. Traces were produced by using a Molecular Dynamics two-dimensional densitometer (courtesy Dr. Thomas Tullius, Department of Chemistry). Ionic strengths were calculated by using the formula $\Gamma = 0.5\sum(M_iZ_i^2)$ where M_i represents the molarity of each ion and Z_i represents the net charge of that ion. The approximate molecular weight of each cross-linked product was determined by using a standard curve based on the known molecular weights of monomeric H3 and H4; the use of standard molecular weight markers is inappropriate in this context due to the anomalous migration of the histones in this gel system (Thomas, 1989).

Analytical Ultracentrifugation. Meniscus depletion sedimentation equilibrium runs were performed in a Beckman Model E analytical ultracentrifuge equipped with Rayleigh interference optics following procedures outlined in Godfrey et al. (1980). Briefly, all tetramer solutions were dialyzed overnight to assure dialysis equilibrium. Double-sector cells with 12-mm aluminum-filled epon centerpieces and quartz windows were filled to a column height of 2.7 mm (100 μL). Sedimentation was performed at 30000 rpm for 24 h using a Beckman An-F rotor. Plates were read on a Nikon microcomparator, and apparent number-, weight-, and z-average molecular weights were calculated by the method of Roark and Yphantis (1969) on a Hewlett-Packard minicomputer (courtesy of Drs. William Harrington and Michael Rodgers, Department of Biology). Modified two-species plots were generated (Elias & Bareiss, 1967), and molecular weight data were subsequently fit to dimer–tetramer, dimer–tetramer–octamer, and isodesmic assembly schemes. The two-species plots and statistics from the fits were used to deduce the probable mode of assembly in each case. All fitting computations were performed on a MicroVAX II using FORTRAN programs of the author's design.

Results

Circular Dichroism. CD spectra of equimolar mixtures of H3 and H4 were recorded to determine whether significant changes in secondary structure occur in response to changes in ionic strength and whether these changes, if any, are the function of a *specific ion* in solution. The results of these secondary structural determinations are presented in Figure 1 and Table I. Increasing amounts of sodium phosphate, sodium sulfate, or ammonium sulfate had only a small (negligible) effect in promoting the formation of α-helices or β-structures; for these solvents, the α-helical content increased from 28% to 31%, while the degree of β-structure varied between 6% and 9%.

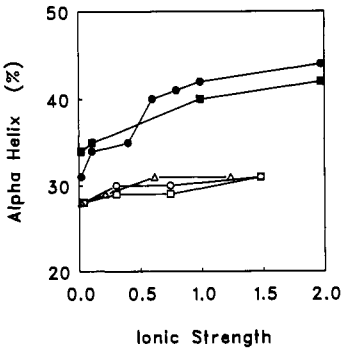

FIGURE 1: α-Helical content of chicken erythrocyte histones H3 and H4 as a function of ionic strength. Secondary structural determinations are based on reference spectra of five proteins of known secondary structure (Chen et al., 1974). (□) Ammonium sulfate; (○) sodium sulfate; (△) sodium phosphate; (■) ammonium chloride; (●) sodium chloride.

Table I: Estimates of Secondary Structure of Chicken Erythrocyte $(H3–H4)_2$ Tetramer from Circular Dichroism Spectra

solvent[a]	$[\theta]_{222}$[b]	% α[c]	% β[c]	$\pm[\theta]_\lambda$[d]
0.01 M NaCl	−8324	31	7	335
0.01 M NaCl	−9421	34	6	268
0.40 M NaCl	−9641	35	7	617
0.60 M NaCl	−11053	40	9	747
0.80 M NaCl	−11653	41	9	595
1.0 M NaCl	−11699	42	4	265
2.0 M NaCl	−12230	44	4	248
0.01 M sodium phosphate	−7480	28	8	393
0.10 M sodium phosphate	−7796	29	8	489
0.25 M sodium phosphate	−8378	31	7	434
0.50 M sodium phosphate	−8378	31	7	394
0.01 M Na_2SO_4	−7327	28	6	474
0.10 M Na_2SO_4	−8161	30	9	356
0.25 M Na_2SO_4	−8112	30	8	314
0.50 M Na_2SO_4	−8324	31	7	425
0.01 M $(NH_4)_2SO_4$	−7529	28	9	383
0.10 M $(NH_4)_2SO_4$	−7895	29	9	379
0.25 M $(NH_4)_2SO_4$	−7845	29	9	342
0.50 M $(NH_4)_2SO_4$	−8378	31	7	413
0.01 M HN_4Cl	−9587	34	7	494
0.10 M NH_4Cl	−9641	35	8	510
1.0 M NH_4Cl	−11116	40	6	447
2.0 M NH_4Cl	−11748	42	6	447

[a] NaCl, Na_2SO_4, $(NH_4)_2SO_4$, and NH_4Cl solvents also contained 10 mM HEPES, pH 7.5, and 1 mM EDTA. Sodium phosphate solutions also contained 1 mM EDTA at pH 7.5. All spectra were recorded at 20.0 ± 0.1 °C. [b] Molar ellipticities at 222 nm where the α-helix exhibits a minimum (Chen et al., 1974). Units are degrees centimeter squared per decimole. [c] Secondary structure estimates are percent of total residues in the indicated conformation, based on reference spectra from α-helix, β-sheet, and random-coil derived from CD spectra of five globular proteins of known structure (Chen et al., 1974). [d] Standard deviation based on residuals between experimental and fitted spectra at 12 wavelengths between 207 and 240 nm, inclusive. Units are degrees centimeter squared per decimole.

On the other hand, equimolar mixtures of H3 and H4 dissolved in chloride-containing solutions (NaCl and NH_4Cl) exhibited a marked increase in secondary structure in response to the levels of chloride ions in solution (Figure 1, Table I). The individual CD spectra show more pronounced valleys at 222 and 208 nm (data not shown), wavelengths where pure α-helices exhibit their minima (Adler et al., 1973); the largest changes in these minima occur between 0.4 and 0.6 M NaCl. In the case of H3–H4 mixtures dissolved in NaCl, there was a net increase in α-helical content of 13% accompanied by a small (3%) decrease in β-structure. For H3 and H4 dissolved in NH_4Cl, the α-helical content increased by 8%, while the β-content remained relatively stable. The spectra obtained

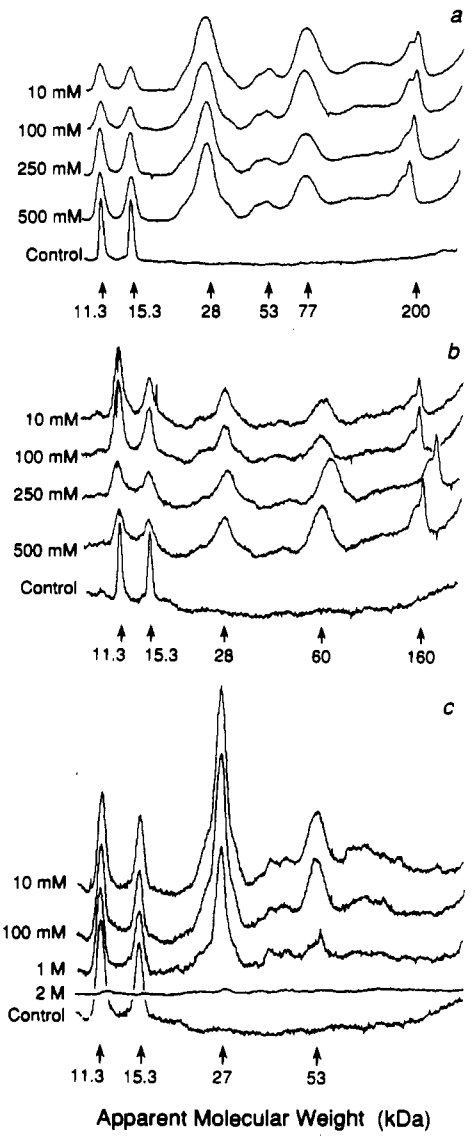

FIGURE 2: Chemical cross-linking of chicken erythrocyte histones H3 and H4 using DSP. Histones were dissolved in (a) 10–500 mM sodium phosphate, (b) 10–500 mM sodium sulfate, or (c) 10 mM–2 M sodium chloride. Control lanes represent unreacted H3 and H4. Size markings beneath each series of tracings are calculated as described by Thomas (1989); see Experimental Procedures for details. The amount of protein applied to each of the lanes was adjusted for the *qualitative* identification of all cross-linked products.

in NaCl over the ionic strength range examined were similar to those obtained in NH_4Cl, while the spectra obtained in Na_2SO_4 were similar to those obtained in $(NH_4)_2SO_4$. Therefore, the Na^+ and NH_4^+ counterions did not seem to have a significant effect on the CD spectra generated in these experiments.

Chemical Cross-Linking. Equimolar mixtures of chicken erythrocyte histones H3 and H4 that had been dialyzed into sodium phosphate (10–500 mM), sodium sulfate (10–500 mM), or sodium chloride (10 mM–2 M) solutions at neutrality were subjected to cross-linking by DSP and electrophoresed in order to trap and directly visualize the minimally sized predominant assemblies between the two polypeptides. In sodium phosphate (Figure 2a), four major cross-linked products are observed (28, 53, 77, and ~200 kDa) regardless of the concentration of phosphate ions in solution. The 28- and 53-kDa products roughly correspond to the H3–H4 heterodimer (27.7 kDa) and the $(H3–H4)_2$ tetramer (53.2 kDa), respectively. The other oligomeric products most likely rep-

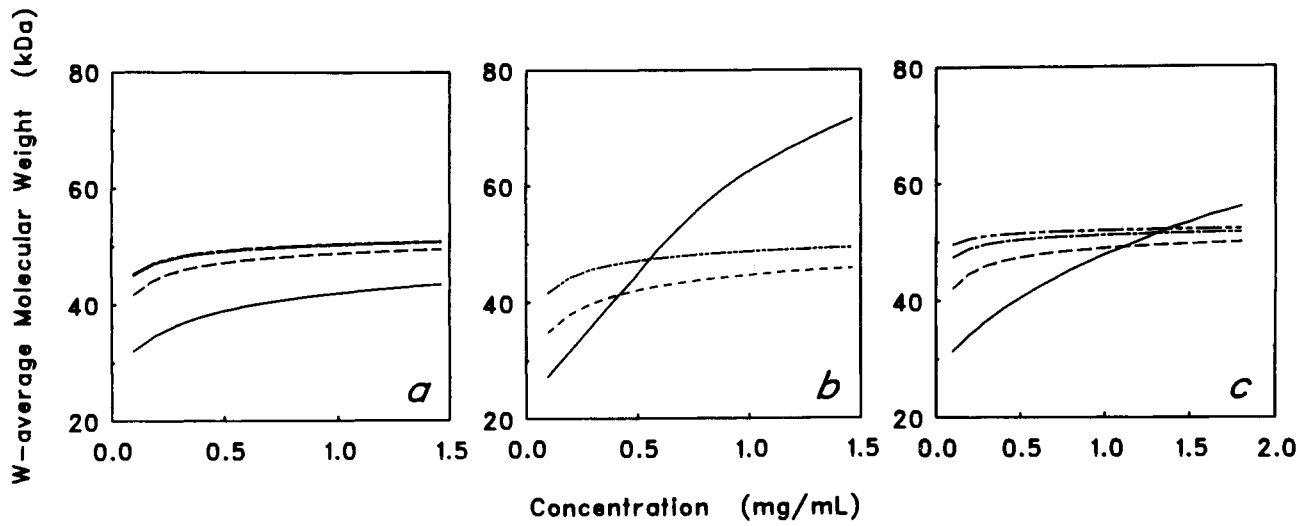

FIGURE 3: Meniscus depletion sedimentation of H3 and H4 in various ionic environments. Statistical goodness-of-fit for each experiment is presented in Table II. (a) H3 and H4 in 100 mM (—), 500 mM (– –), 1 M (-·-), and 2 M NaCl (-···). (b) H3 and H4 in 100 (—), 250 (– –), and 500 mM (-···) sodium phosphate. (c) H3 and H4 in 100 (—), 140 (– –), 250 (-·-), and 500 mM $(NH_4)_2SO_4$ (-···).

resent higher order aggregation states which have accumulated over the course of the cross-linking. Whether these oligomers are part of a reversible distribution in true solution or are terminal aggregation states cannot be deduced solely by this experiment. In sodium sulfate (Figure 2b), three predominant products are observed: 28 kDa (H3–H4), 60 kDa [$(H3–H4)_2$], and ~160 kDa (aggregated oligomer). Little dependence on ionic strength is observed here, as was the case for the phosphate series, above.

In sodium chloride, the observed interactions are substantially different from those observed in sulfate or phosphate (Figure 2c). At low ionic strength, 27- and 53-kDa cross-linked products are favored and, as the ionic strength is raised, increased aggregation is observed in the form of higher molecular weight products until, at 2 M NaCl, the products of the cross-linking reaction are too large to enter the gel.

The cross-linking reactions were not performed on histones dissolved in the corresponding ammonium salts since the solvent competes with the amino groups of the histones for the reagent.

Equilibrium Sedimentation. Equimolar mixtures of chicken erythrocyte histones H3 and H4 were dialyzed into sodium phosphate (100–500 mM), ammonium sulfate (100–500 mM), or sodium chloride (100 mM–2 M) solutions at neutrality and subjected to high-speed (meniscus depletion) equilibrium sedimentation in order to determine minimum molecular weights, in addition to number-average, weight-average, and z-average molecular weight moments as a function of protein concentration within the ultracentrifuge cell.

In all concentrations of sodium chloride, the distribution of mass ranged from ~27 to ~53 kDa. In this case, the system seems to obey an H3–H4 dimer–$(H3–H4)_2$ tetramer equilibrium scheme (Table II; Figure 3a). The equilibrium constant K_1, which describes the H3–H4 dimer–$(H3–H4)_2$ tetramer reaction, increases in response to the amount of chloride in solution. This can be seen qualitatively in Figure 3a, where the w-average molecular weight moments become more homogeneous with respect to concentration; the equilibrium is highly shifted toward the $(H3–H4)_2$ tetramer regardless of protein concentration. Large values for the z-average molecular weights at relatively low protein concentration (~0.8 mg mL^{-1}) indicate that some aggregation is occurring in these chloride-containing solvents (data not shown). Despite this and the higher order products observed during DSP cross-linking, the aggregation of *free* H3–H4 was not large enough to suggest a significant isodesmic mode of assembly with large numbers of higher order products *in the equilibrium.*

Table II: Equilibrium Constants for the Higher Order Assembly of H3 and H4 in Various Ionic Environments

solvent[a]	assembly scheme[b]	K_1[c] (L mol^{-1})	K_2[d] (L mol^{-1})	ΔM_n[e]	ΔM_w[e]
100 mM $(NH_4)_2SO_4$	D–T–O	6.24×10^4	8.51×10^4	0.8	0.3
140 mM $(NH_4)_2SO_4$	D–T	8.76×10^5		1.0	2.0
250 mM $(NH_4)_2SO_4$	D–T	4.32×10^6		0.3	0.5
500 mM $(NH_4)_2SO_4$	D–T	1.25×10^7		1.6	1.0
100 mM NaCl	D–T	8.43×10^4		1.2	1.4
500 mM NaCl	D–T	7.96×10^5		1.3	2.9
1.0 M NaCl	D–T	1.92×10^6		1.3	2.9
2.0 M NaCl	D–T	2.06×10^6		0.6	2.2
100 mM sodium phosphate	D–T–O	9.25×10^2	1.25×10^5	1.0	2.1
250 mM sodium phosphate	D–T	1.72×10^5		0.6	1.4
500 mM sodium phosphate	D–T	7.81×10^5		1.0	1.1

[a] NaCl and $(NH_4)_2SO_4$ solvents also contained 10 mM HEPES, pH 7.5, and 1 MM EDTA. Sodium phosphate solutions also contained 1 mM EDTA at pH 7.5. All spectra were recorded at 20.0 ± 0.1 °C. [b] D–T, H3–H4 dimer–$(H3–H4)_2$ tetramer; D–T–O, H3–H4 dimer–$(H3–H4)_2$ tetramer–$2(H3–H4)_2$ octamer. [c] Equilibrium constant for dimer–tetramer association. [d] Equilibrium constant for tetramer–octamer association. [e] Standard deviation based on the residuals between the experimental and calculated molecular weights ($\times 10^{-3}$), as a measure of goodness-of-fit.

At 100 mM sodium phosphate, pH 7.5, the mass distribution indicates species ranging from ~27 to >70 kDa (Figure 3b). In this case, the behavior of H3 and H4 is best described by an H3–H4 dimer–$(H3–H4)_2$ tetramer–$2(H3–H4)_2$ octamer assembly system (Table II). Values for K_1 and K_2 indicate that the dimer–tetramer equilibrium is not massively shifted toward the tetramer but the formation of an $2(H3–H4)_2$ octamer from an $(H3–H4)_2$ tetramer is greatly favored once the tetramer is formed. At phosphate concentrations of 250 mM and above, the data suggest an equilibrium between the H3–H4 dimer and the $(H3–H4)_2$ tetramer. Both Figure 3b and the equilibrium constants in Table II indicate that the H3–H4

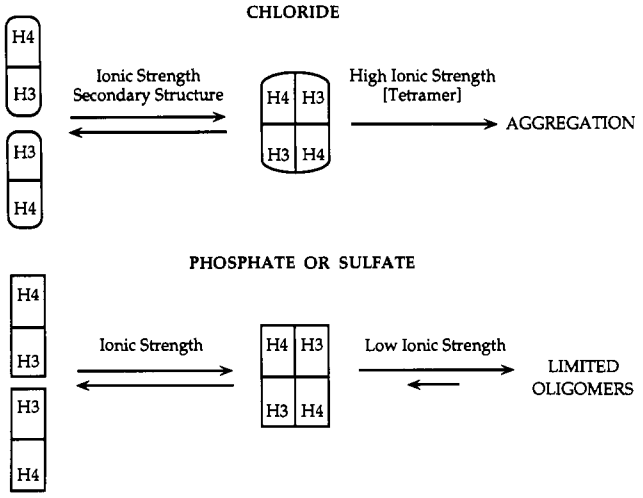

FIGURE 4: Summary of circular dichroism, chemical cross-linking, and equilibrium sedimentation data on the behavior of H3–H4 in various chloride-, phosphate-, and sulfate-containing solvents. In solutions containing chloride, increases in ionic strength are accompanied by an increase in α-helicity, and the equilibrium between the H3–H4 dimer and $(H3–H4)_2$ tetramer is shifted toward the tetramer species. While increases in phosphate and sulfate concentration also shift the equilibrium toward the $(H3–H4)_2$ tetramer, there is no accompanying increase in secondary structure. Depending upon the ionic environment, limited oligomers or large aggregates can be trapped by the cross-linking reagent DSP.

dimer–$(H3–H4)_2$ tetramer equilibrium is shifted to the right at higher ionic strengths, as was the case with H3 and H4 in sodium chloride.

The behavior of H3–H4 in ammonium sulfate is similar to its behavior in sodium phosphate. In 100 mM ammonium phosphate, protein species from ~30 to >53 kDa are oberved, and the data are best fit to an H3–H4 dimer–$(H3–H4)_2$ tetramer–$2(H3–H4)_2$ octamer assembly scheme (Figure 3c; Table II). Above 140 mM $(NH_4)_2SO_4$, a mass distribution from 40 to 53 kDa is observed, and the data in these higher concentrations of sulfate are best fit to an H3–H4 dimer–$(H3–H4)_2$ tetramer assembly scheme (Figure 3c, Table II). This is borne out by plots of M_z vs C over the ammonium sulfate concentration range examined, which show that aggregation (albeit limited and only seen at very high protein concentrations) is occurring at lower sulfate concentrations and that this aggregation does not occur at all at higher sulfate concentrations, where values of M_w and M_z vs C are similar (data not shown). In any case, K_1 increases 1000-fold when the sulfate concentration is increased from 100 to 500 mM, with the equilibrium greatly shifted toward the $(H3–H4)_2$ tetramer at the higher ionic strengths (Figure 3c, Table II). For H3–H4 in 100 mM ammonium sulfate, K_2 is slightly larger than K_1, accounting for the shape of its plot in Figure 3c.

Summary of Results. A schematic summary of the circular dichroism, chemical cross-linking, and equilibrium sedimentation experiments is presented in Figure 4. In chloride-containing solutions, the equilibrium between an H3–H4 dimer and an $(H3–H4)_2$ tetramer is responsive to changes in ionic strength and paralleled by changes in α-helicity. Extensive aggregation is promoted at higher protein concentrations, especially at high levels of NaCl. In sulfate- or phosphate-containing solutions, the equilibrium point is again governed by ionic strength, but there are no significant changes in α-helicity accompanying shifts in the equilibrium. Small oligomers can also be formed in low ionic strength sulfate- or phosphate-containing solutions and can be trapped by the cross-linking reagent; these oligomers are much smaller than those formed in the presence of sodium chloride. It appears that, under most conditions, the equilibrium exists between an H3–H4 dimer and an $(H3–H4)_2$ tetramer; this equilibrium is highly dependent upon ionic strength and, more importantly, appears to be ion-specific.

Discussion

By probing the behavior of equimolar mixtures of histones H3 and H4 in solution using three fundamentally different physical and chemical techniques, it appears that the assembly of H3–H4 dimers into higher order species and the extent of this assembly are dependent upon the specific counterions present in solution as well as the total ionic strength of the solution. When histones H3 and H4 are dissolved in sodium chloride solutions, an increase in the ionic strength of the solution leads to an increase in α-helical content (Figure 1, Table I), which is in turn paralleled by an increase in the equilibrium constant K_1 [K_1 describes the equilibrium between H3–H4 dimers and $(H3–H4)_2$ tetramers; Table II]. While the interaction of H3 and H4 in high concentrations of sodium chloride is best described by an H3–H4 dimer–$(H3–H4)_2$ tetramer assembly scheme, z-average molecular weights in excess of 53 000 were observed, especially at higher concentrations of sodium chloride (data not shown). The propensity of the tetramers to aggregate in high concentrations of NaCl may be further compounded by the cross-linking reagent, causing the formation of higher molecular weight complexes which are not free to dissociate back to the dimeric or tetrameric forms. (It is important to note that the cross-linking results provide no information regarding the extent or direction of equilibria between individual interacting species; rather, the cross-linking reaction traps all reactive species which appear in the solution over the course of the experiment.) In this light, even though large aggregates are found upon cross-linking, the sedimentation studies show that in sodium chloride solutions of 500 mM or more, the $(H3–H4)_2$ tetramer is the predominant protein complex *in the distribution* while at 100 mM NaCl there is more of a mixed population of H3–H4 dimers and $(H3–H4)_2$ tetramers in solution.

In both phosphate- and sulfate-containing solutions, little ionic strength dependence is seen in the secondary structural analysis (Table I). Despite this, K_1 does increase as the ionic strength of the environment is increased, regardless of the assembly scheme used to describe the system (Table II). Electrophoretic analysis of cross-linked products indicates that H3–H4 dimers, $(H3–H4)_2$ tetramers, and several limited oligomers coexist in solution but unlike the chloride case the equilibrium data indicate that only a small amount of aggregation is taking place. In these solvents, the small oligomers may be in true equilibrium with the dimeric and tetrameric species, so under none of the phosphate or sulfate conditions does one notice the formation of cross-linked products that are so large that they cannot enter the gel matrix (Figure 2a,b). For H3 and H4 in both phosphate and sulfate, the equilibrium again exists primarily between an H3–H4 dimer and an $(H3–H4)_2$ tetramer, with the ionic strength governing the balance of the equilibrium (Figure 3, Table II).

The changes in K_1 observed in this study with respect to ionic strength are fairly similar for each of the solvent systems. Along with the shift away from the H3–H4 dimer toward the $(H3–H4)_2$ tetramer, H3 and H4 in chloride undergo a large structural rearrangement, with a net gain of 8–13% α-helix, while H3 and H4 in phosphate or sulfate do not undergo any significant net secondary structural rearrangements at all (Table I). It appears that "less structured" molecules are still capable of forming a tetrameric species. Possible explanations

for this behavior are that the observed increase in the α-helical content of H3 and H4 in the presence of chlorides is in regions of H3 and H4 away from the interface, that the interface is primarily composed of residues in β-sheet or random-coil conformation, or that the proteins may be responding to the ionic species in solution by binding with one another, thereby hiding certain surface regions from the solution whose exposure has become unfavorable. Although there is no *net* change in secondary structure for H3–H4 in phosphate or sulfate, the observation that H3 and H4 can form limited oligomers in low concentrations of these salts may be due to the presence of certain "sticky" hydrophobic regions on the surface of the H3–H4 complex which do not exist at higher ionic strengths.

Overall, our results are consistent with those reported by Roark et al. (1974), who examined H3 and H4 *in the absence of H2A and H2B*. Their work was performed in an acetate/bisulfite buffer at extremely low ionic strength conditions ($\Gamma \sim 0.1$, pH 5.0); they find that there is a "concentration-dependent reversible equilibrium" between an H3–H4 dimer and its corresponding tetramer, the equilibrium being shifted toward the tetramer. With regard to work favoring a tripartite organization of the histone octamer (Thomas & Kornberg, 1975; Thomas & Butler, 1977; Eickbush & Moudrianakis, 1978; Godfrey et al., 1980; Burlingame et al., 1985), that is, two H2A–H2B dimers flanking a central $(H3–H4)_2$ tetramer, comparison is not as straightforward since these earlier studies employed whole core histone octamers $[(H2A–H2B)_2(H3–H4)_2]$ free in solution or in chromatin; however, all of these studies were performed in very high ionic strength solutions (2 M NaCl or 2.6 M ammonium sulfate) where, on the basis of results from the present study, one would expect that H3 and H4 would exist primarily as a $(H3–H4)_2$ tetramer. The work of Eickbush and Moudrianakis (1978), which examined the stability of the octamer under a variety of environmental conditions, indicates that the octamer is the predominant protein assembly in high ionic strength solutions and at high protein concentrations; however, this tripartite assembly persists even under less favorable conditions [cf. Godfrey et al. (1980)]. The present work would support the existence of $(H3–H4)_2$ as a stable entity under these conditions.

There have been several studies which were interpreted to support an alternative model for the organization of the histone core octamer, i.e., its dissociation into two H2A–H2B–H3–H4 (heterotypic) tetramers. The light-scattering studies of Campbell and Cotter (1976) were performed in high salt at pH 9 and used extinction coefficients substantially less (~25%) than those employed by us (Eickbush & Moudrianakis, 1978; Godfrey et al., 1990) and by Thomas and Butler (1977). Since the molecular weight obtained through light-scattering measurements is inversely dependent on protein concentration (Cantor & Schimmel, 1980), which is estimated spectroscopically, the resultant molecular weight may have been substantially underestimated. Furthermore, due to the protein concentration at which the experiments were performed, the protein distribution is shifted away from the core octamer and toward the H2A–H2B dimer and $(H3–H4)_2$ tetramer (Eickbush & Moudrianakis, 1978), and it appears that the lower molecular weight H2A–H2B dimer was simply not detected. [Both the "heterotypic tetramer" and the $(H3–H4)_2$ tetramer would have molecular weights similar to the 55-kDa species observed in the experiment.]

The sedimentation studies of Weintraub et al. (1975) and Chung et al. (1978) claimed the presence of a stable species with the apparent molecular weight of a heterotypic tetramer in 2 M NaCl, pH 9. The partial specific volumes used in both studies are substantially less than those employed in studies from this laboratory and by Thomas and Butler (1977), and this leads to an underestimation of the apparent molecular weights on the order of 10–15%. More importantly, these sedimentation experiments were performed at relatively low rotor speeds, and this would make the detection of low molecular weight solutes, such as an H2A–H2B or H3–H4 dimer, more difficult [cf. Godfrey et al. (1980)]. As noted above, the distribution of histone species at pH 9 is shifted toward free H2A–H2B dimers and $(H3–H4)_2$ tetramers, so the observed assembly in these studies may simply be the 53-kDa $(H3–H4)_2$ tetramer, with the H2A–H2B dimer going undetected. Using physical techniques similar to those employed by Weintraub et al. (1975) and Chung et al. (1978) as well as meniscus depletion equilibrium sedimentation, we found the molecular weight of the histone complex in 2 M NaCl at neutrality to be 108 000 (Eickbush & Moudrianakis, 1978; Godfrey et al., 1980), implying that two of each of the histone polypeptides are involved in the formation of a stable, octameric complex. Finally, it is interesting to note that the Weintraub group reported that dimers, tetramers, hexamers, and octamers can be produced by cross-linking in the presence of DNA, while time-course cross-linking experiments by Chung et al. (1978) on free histones indicate that a significant proportion of these histone proteins exist as dimers and tetramers as well as octamers.

What is, then, the physiological significance of these findings with respect to the regulation of chromatin structure and, in turn, histone–histone interactions? When bound to the DNA, which is an environment of extremely high phosphate density, the core histones exist as an octameric assembly. (In this type of environment, H3 and H4 would exist as a tetramer even in the absence of the H2A–H2B dimer.) Upon extraction of the histones from the DNA under high salt conditions, the histone assembly remains intact as an octamer. At this point, two theoretical possibilities exist for the dissociation of the subunits which comprise the core histone octamer: either the H2A–H2B dimers can dissociate from the central $(H3–H4)_2$ tetramer before the tetramer can further dissociate into H3–H4 dimers, or the octamer can divide into two symmetrical H2A–H2B–H3–H4 tetramers which then dissociate into H2A–H2B and H3–H4 dimers. Gel filtration (Eickbush & Moudrianakis, 1978), sedimentation equilibrium (Godfrey et al., 1980), high-precision osmometry (Stein & Page, 1980), fluorescence and circular dichroism (Butler & Olins, 1982), and isothermal calorimetry studies (Benedict et al., 1984) all argue for the separation of the H2A–H2B dimers away from the $(H3–H4)_2$ tetramer as being the *first* step in the dissociation of the core octamer and that these subunits are stable entities in a variety of pH, ionic strength, and concentration conditions. No studies have provided any kind of evidence in favor of the formation of a heterotypic tetramer as the *first* (i.e., energetically favored) step in the dissociation of the core octamer. The H2A–H2B dimer–$(H3–H4)_2$ tetramer interface appears to be the more "open" and easily solvated channel in the model of Burlingame et al. (1985). While the $(H3–H4)_2$ tetramer has a bilobal appearance, no obvious solvent channel is present within it which can be envisioned as responsive to changing environmental conditions so as to split the tetramer in half. In solution and in the absence of DNA, it appears thermodynamically much more favorable to *first* remove the H2A–H2B dimer from the $(H3–H4)_2$ tetramer than it is to divide the $(H3–H4)_2$ tetramer in half. In considering these experimental findings, one must be mindful of the natural partners of H3 and H4 in the cell. With respect to the

H2A–H2B dimer, its presence may sharply influence the ability of H3 and H4 to exist as an independent heterodimer. Furthermore, its well-characterized association with the $(H3–H4)_2$ tetramer indicates that the H2A–H2B dimer could prevent small-scale aggregation of H3 and H4 by serving as a molecular "cap", i.e., by binding to the sites available on either side of the $(H3–H4)_2$ tetramer, the H2A–H2B dimer may be blocking tetramer "sticky" regions responsible for self-aggregation. No conclusions can be drawn as yet as to how the local association of the DNA double helix with the histones will influence the distribution of histone species during the opening up of the nucleoprotein filament. While the vast majority of studies on histone–DNA complexes show that the "canonical nucleosome" contains two H2A–H2B dimers and one $(H3–H4)_2$ tetramer, there is a report of an in vitro reconstituted nucleosome-like particle with an altered stoichiometry; this particle contains two copies of histones H2A and H2B but only one each of histones H3 and H4. Whether this assembly reflects the existence of an alternate assembly pathway or is a result of specific differences in reconstitution procedures remains to be determined [see Ellison and Pulleyblank (1983) and references contained therein].

On the basis of this and other studies, we conclude that under most physiological conditions and in the absence of DNA, histones H3 and H4 exist as a stable $(H3–H4)_2$ tetramer. Furthermore, H3 and H4 *can* exist as a stable H3–H4 dimer, its equilibrium with the $(H3–H4)_2$ tetramer being both ion-specific and ionic strength dependent. These observations support the concept of the core histone octamer having a tripartite organization in solution, with the two H2A–H2B dimers flanking a central $(H3–H4)_2$ tetramer complex, rather than being organized as two identical heterotetramers. The H2A–H2B dimers may act as molecular "caps" in this assembly, preventing aggregation of the $(H3–H4)_2$ tetramer and thus aiding in the formation of the tripartite octamer.

Registry No. Chloride, 16887-00-6; sulfate, 14808-79-8; phosphate, 14265-44-2.

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