Effect of Aggregation of Histone Octamers in High-Salt Solutions on Circular Dichroism Spectra†

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ABSTRACT: The circular dichroism (CD) of freshly prepared chicken erythrocyte core histones has been reexamined in high concentrations of ammonium sulfate and sodium chloride, conditions which cause drastic changes in the solubility and aggregative properties of these proteins. After sample clarification by ultracentrifugation, no significant net changes are detected in the secondary structure of the core histones in the range of 2.0–2.5 M ammonium sulfate. There is also no significant difference between the CD spectra of histone solutions in 2 M sodium chloride and clarified solutions of histones in high concentrations of ammonium sulfate. It was observed that sample clarification by ultracentrifugation immediately prior to taking CD spectra was necessary for signal stabilization, especially under conditions which begin to favor crystallization of the histones.

The DNA of eukaryotic cells is structurally organized by tightly bound histone octamers into flexible strings of repeating arrays of nucleosomes (Kornberg, 1974) which can condense into higher order chromatin structures. Biophysical studies [e.g., see reviews by Allfrey (1980) and Fasman (1978)] have shown that small changes in ionic strength can cause changes in chromatin conformation, and different ionic environments can cause perturbation of the structure. Several circular dichroism studies have been performed to determine the extent to which the secondary structure of core histones is sensitive to changes in ionic strength in solution. For example, Bidney and Reeck (1977) observed that at low NaCl (10 mM) concentrations, the core histones maintained a highly irregular conformation and that raising the NaCl concentration to 2 M led to the formation of substantial amounts of α-helix. Also, Olins et al. (1977) determined that the α-helical content of core histones increases significantly from 0 to 1 M NaCl and remains essentially constant from 1 to 2 M NaCl. However, Prevelige and Fasman (1987) demonstrated unequivocally that the helical content of the histone octamer increased significantly upon changing from 1 to 2 M NaCl.

More recently, a preliminary neutron scattering study claimed to have documented an increase in particle dimensions for the octamer complex in going from 2 M NaCl to 3.5 M ammonium sulfate (Uberbacher & Bunick, 1985). These authors suggested that this putative "expansion" in the length of the core histone octamer was the explanation for the difference between the length of the nucleosome core particle determined by Richmond et al. (1984) and that of the core histone octamer determined by Burlingame et al. (1985) by the use of X-ray crystallography. This proposal was tested in a more recent circular dichroism study which examined the differences in secondary structure of core histones as a function of ammonium sulfate concentration (Park & Fasman, 1987). It was found that the response of the core histones was complex, with the molar ellipticity of the complex increasing from 2.0 to 2.2 M ammonium sulfate, decreasing from 2.2 to 2.3 M ammonium sulfate, and again increasing from 2.3 to 2.5 M ammonium sulfate, a behavior substantially different than that for histones in high concentrations of sodium chloride and for proteins in general (Ladner et al., 1977; Prevelige & Fasman, 1987). In the course of performing similar optical rotatory dispersion and circular dichroism experiments on core histone proteins (Godfrey et al., 1990), it was observed that in the high ammonium sulfate buffers used in these studies, concentrations of which approach the octamer crystallization condition (Burlingame et al., 1985), significant nucleation, aggregation, and precipitation of the protein occur. This behavior leads to a significant increase in the light scattering properties of the samples, and this can have an adverse effect on the observed circular dichroism (CD)[1] spectra (Urry & Krivacic, 1970). Given the problematic nature of handling the histones under these conditions, we decided jointly to reexamine the properties of the core histones stored at 4 °C or at −70 °C in high ammonium sulfate and sodium chloride buffers in order to find conditions which controlled histone aggregation so that the CD signal observed was a true, stable signal for histones existing in true solution.

Materials and Methods

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

Preparation of Histones. Histones were prepared by two essentially similar salt extraction methods, i.e., either the method of Eickbush and Moudrianakis (1978; method I) or that of Ramsay-Shaw et al. (1976; method II) as has been

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[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; DTT, 1,4-dithiothreitol (Cleland's reagent).

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customary in the Johns Hopkins and Brandeis laboratories, respectively. Histones produced by method I were stored as concentrated solutions (>10 mg/mL) at 4 °C in 2 M NaCl, 10 mM HEPES, and 1 mM EDTA, pH 7.5. Histones produced by method II were stored frozen at −70 °C in solutions containing 10 mM sodium phosphate, 0.25 mM Na_2EDTA, 0.1 mM DTT, and 0.1 mM PMSF, pH 7.2. The histones are exposed to 1 mM PMSF throughout both isolation procedures, but the PMSF is dialyzed out of histone solutions prepared by method I before storage. Only freshly prepared histones (no older than 10 days) were used for CD measurements. It is necessary that frozen histones, once thawed, undergo an extensive dialysis (at least 48 h) in order for them to renature to a steady state (data not shown).

Circular Dichroism Measurements. Stock chicken erythrocyte histones were diluted approximately 20-fold into solvents ranging from 2.0 to 2.5 M ammonium sulfate, each containing 10 mM sodium pyrophosphate and 0.25 mM EDTA, pH 7.2, to yield a histone concentration in the range 0.2–0.6 mg/mL. The diluted histones were then dialyzed against the diluent for 48 h. It was found that the histone stock solution must be diluted prior to dialysis against high concentrations of ammonium sulfate; otherwise, the protein will precipitate. Samples were removed from dialysis and centrifuged in a Beckman 50TI or 70.1TI rotor at 30000 rpm for 30 min. After clarification, the supernatant was carefully pipetted off, taking care to not disturb any small pellet which may be present. Circular dichroism measurements were made in a Jasco J-500C spectropolarimeter (courtesy of Dr. Yong Shin, National Institute on Aging, Gerontology Research Centre, Baltimore, MD) or in a Jobin-Yvon Mark V autodichrograph. All measurements were made in 0.05-, 0.1-, or 0.2-cm path-length cells (Hellma Scientific) at 20.0 ± 0.1 °C. The temperature was regulated by a Lauda K-2/R refrigerated bath circulator (Brinkmann). For each sample, the ellipticities were taken in two different ways: by direct reading at 12 or 13 manually set wavelengths (204 or 207–240 nm at 3-nm intervals) after the signals had stabilized for at least 30 s, the rationale for this procedure being given elsewhere (Godfrey et al., 1990), as well as by automatic manipulation in steps of 0.2 nm, as described in Park and Fasman (1987), for comparison. The molar ellipticities, $[\theta]_\lambda$, were calculated in units of degrees centimeter squared per decimole, using 111.1 as the molecular weight of an average amino acid residue.

The absorbance of the sample at 277 and 329 nm was determined in black-walled 1-cm path-length quartz cuvettes (Hellma Scientific) as described by Godfrey et al. (1990) just prior to taking CD measurements. CD samples were tested for purity by polyacrylamide gel electrophoresis as described in Godfrey et al. (1990).

Selective Precipitation of Histones. The possibility of selective precipitations of the subclasses of the histone octamer in high-salt environments was monitored by using acid–urea–Triton polyacrylamide gels. Histone solutions in specific concentrations of ammonium sulfate were made by dialysis using previously washed dialysis tubing (Spectrapor 3, Spectrum Medicals, Inc.). The dialysis was performed with histone stock solutions of less than 1 mL against 1 L of dialysis buffer which contained 10 mM sodium pyrophosphate, 0.25 mM EDTA, and 0.1% 2-mercaptoethanol in addition to ammonium sulfate at the required concentration. The dialysis proceeded for 48 h with two changes of dialysate. Samples were removed from dialysis and centrifuged in a Beckman 70.1TI rotor at 30000 rpm for 30 min. After clarification, the supernatant was carefully pipetted off, taking care not to disturb any small pellet which may be present. Supernatants were subsequently electrophoresed on acid–urea–Triton polyacrylamide gels [15% acrylamide, 0.1% methylenebis(acrylamide), 1 M acetic acid, 8 M urea, and 8 mM Triton X-100]. This method is known for its high resolution of those histone subclasses induced by species variation, sequence variation, and posttranslational modification (Bonner et al., 1980; Waterborg & Harrington, 1987). The gel was silver stained with Bio-Rad silver staining reagents according to the manufacturer's manual.

Turbidity Measurements. In the high concentrations of ammonium sulfate (2.0–2.5 M) used in some experiments, the precipitation range of the histones is approached, and thus there is some increase in the scattering properties of the sample, which may have an unpredictable effect on the CD measurements. For this reason, experiments were performed to determine the extent of aggregation under certain solvent conditions. (1) Stock chicken erythrocyte histone solutions (5 mg/mL) in 2 M NaCl, 10 mM HEPES, and 1 mM EDTA, pH 7.5, were clarified by ultracentrifugation at 30000 rpm for 30 min in a Beckman 50TI rotor. After clarification and concentration determination, the stock histones were diluted 10-fold into the 2 M NaCl buffer described above or into 2.3 M ammonium sulfate, 10 mM sodium pyrophosphate, and 0.25 mM EDTA, pH 7.2. Lang-Levy volumetric pipets were used to accurately perform the dilutions. Changes in absorbance at 277 and 329 nm with respect to time of samples which had or had not been clarified again after the 10-fold dilution were then monitored on a Gilford spectrophotometer. (2) In a similar experiment, the stock histones were diluted into the 2.3 M ammonium sulfate buffer and dialyzed for 24 h against that buffer. Absorbances at 277 and 329 nm were then measured, the solution was clarified, and absorbances were measured again. (3) Finally, clarified stock histones (10 mg/mL) in the 2 M NaCl buffer described above were diluted 30-fold into 2.0, 2.2, or 2.4 M ammonium sulfate, each containing 10 mM sodium pyrophosphate and 0.25 mM EDTA, pH 7.2. The dilutions were mixed thoroughly, and ellipticities were read at 222 nm in 0.1-cm path-length cells as described above.

RESULTS

Circular Dichroism. CD spectra were recorded for chicken erythrocyte core histones dissolved in solutions of ammonium sulfate (2.0–2.5 M) buffered with 10 mM sodium pyrophosphate and 0.25 mM EDTA, pH 7.2. Spectra of the results obtained using the Jasco J-500C (Figure 1a) and the Jobin-Yvon Mark V autodichrograph (Figure 1b) show that the core histones do not undergo appreciable net changes in secondary structure in this range of ammonium sulfate concentrations. When $[\theta]_{222}$ was used as a direct measure of the α-helical content of the proteins, no change was seen in this value over the range of ammonium sulfate concentrations examined; $[\theta]_{222}$ remained stable at approximately −15000 deg cm^2 dmol^{-1} (Figure 2). There was also no significant difference in secondary structure between histones in 2.0 M NaCl buffer and histones in 2.0–2.5 M ammonium sulfate (Figure 3).

Turbidity Experiments. A clarified 5 mg/mL stock histone solution in 2 M NaCl, 10 mM HEPES, and 0.1 mM EDTA, pH 7.5, was diluted 10-fold into the same buffer, and its absorbance at 329 nm was monitored to measure any scattering due to aggregation of the histone octamers; no aggregation was seen in this case. However, when the same stock solution was diluted into 2.3 M ammonium sulfate, 10 mM sodium pyrophosphate, and 0.25 mM EDTA, pH 7.2, there was a marked absorbance increase at 329 nm; the ratio of absorbance at 329 nm to that at 277 nm was 17% upon ad-

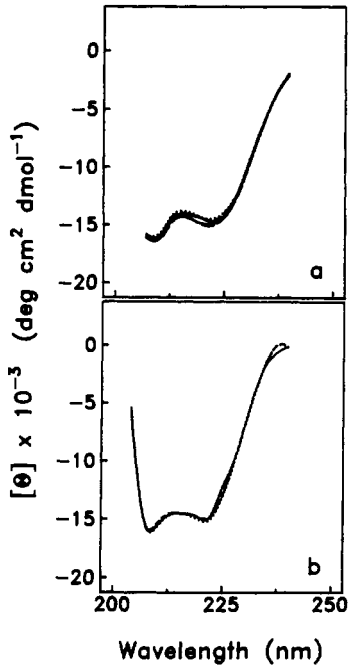

FIGURE 1: Circular dichroism spectra of chicken erythrocyte histones dissolved in varying concentrations of ammonium sulfate. All ammonium sulfate solutions include 10 mM sodium pyrophosphate and 0.25 mM EDTA, pH 7.2. (a) Spectra obtained by using a Jasco J-500C spectropolarimeter in 2.0 (—), 2.1 (···), 2.2 (- - -), 2.3 (-·-), 2.4 (-···-), and 2.5 M (– –) ammonium sulfate. (b) Spectra obtained by using a Jobin-Yvon Mark V autodichrograph in 2.0 (—), 2.2 (···), and 2.4 M (- - -) ammonium sulfate. For both (a) and (b), the histones exhibit almost identical CD behavior for the ammonium sulfate concentrations examined; as such, the five curves in (a) and the three curves in (b) overlap one another to such an extent that they become indistinguishable.

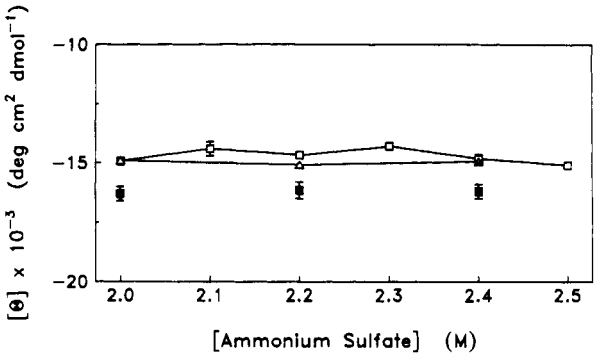

FIGURE 2: Circular dichroism molar ellipticity values at 222 nm ($[\theta]_{222}$) as a function of ammonium sulfate concentration. Samples were prepared by dialysis into an ammonium sulfate buffer containing 10 mM sodium pyrophosphate and 0.25 mM EDTA, pH 7.2, and clarified by ultracentrifugation prior to determination of concentration. CD values were read by using both a Jasco J-500C spectropolarimeter (□) and a Jobin-Yvon Mark V autodichrograph (△). Samples were also prepared by dilution of concentrated stock histones in 2 M NaCl, 10 mM HEPES, and 1 mM EDTA, pH 7.5, into the appropriate ammonium sulfate buffer and read on the Jasco J-500C *without* clarification being performed prior to reading the CD of the sample (■).

dition, rising to 21% after 4 h. When the experiment was repeated but now the diluted histones were clarified prior to measurement to remove any nucleation sites of preaggregated protein, no significant scatter was detectable at 329 nm in either solvent. When a fresh stock of histone octamers was diluted into 2.3 M ammonium sulfate buffer and then dialyzed for 24 h against the same buffer, a significant degree of aggregation was again seen; the ratio of the absorbance at 329 nm to that at 277 nm is 38%, but was reduced to only 1% upon

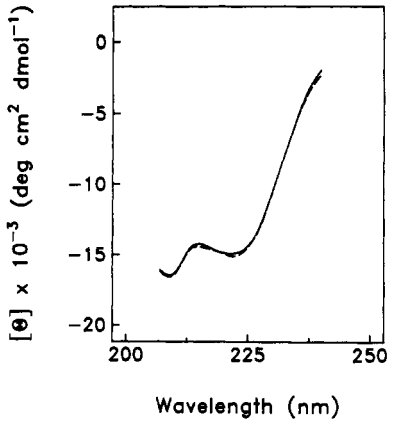

FIGURE 3: Circular dichroism spectra of chicken erythrocyte histones in 2 M NaCl, 10 mM HEPES, and 1 mM EDTA, pH 7.5 (—), and in 2 M $(NH_4)_2SO_4$, 10 mM $Na_4P_2O_7$, and 0.25 mM EDTA, pH 7.2 (- - -). Spectra were obtained by using a Jasco J-500C spectropolarimeter. The histones exhibit almost identical CD behavior for the two solvents examined; as such, the two curves overlap one another to such an extent that they become indistinguishable.

clarification. When the conditions of centrifugation of 20000*g* (12 500 rpm in a Beckman JA-20 rotor) for 30 min (Park & Fasman, 1987) were used, the ratio of absorbances was only reduced to 9%.

CD measurements were done in a similar experiment where concentrated histones (10 mg/mL) were diluted 30-fold into three different buffers ranging from 2.0 to 2.4 M ammonium sulfate. These protein solutions yielded significantly lower molar ellipticity values at 208 and 222 nm than their clarified counterparts (Figure 2). Acid–urea–Triton gels did not indicate that a particular histone isotype was being selectively precipitated by the high concentrations of ammonium sulfate in the solvents (data not shown).

Discussion

In the present study, the effect of high concentrations of ammonium sulfate on histone secondary structure was probed by using circular dichroism spectroscopy. We find that when the solutions are clarified by centrifugation at 30000 rpm for 30 min, the core histones do not undergo any appreciable structural changes in the 2.0–2.5 M ammonium sulfate concentration range. There is also no significant difference in secondary structure between histones in 2.0 M NaCl and histones in 2.0–2.5 M ammonium sulfate. These observations were made on two independently calibrated spectropolarimeters using histones prepared by two similar salt extraction methods (see Materials and Methods).

In the course of preliminary experiments at The Johns Hopkins University monitoring the change in absorbance at 277 and 329 nm of histone solutions in either ammonium sulfate or sodium chloride buffers, it was noted that a significant amount of protein was salting out in the ammonium sulfate buffers but not in the sodium chloride buffer. However, when a histone–ammonium sulfate solution is clarified by ultracentrifugation at 30 000 rpm, the sample remains stable with respect to turbidity for at least 4 h, which is more than enough time to determine CD and absorbance values. Thus, when all of the protein samples were clarified by centrifugation at 30 000 rpm before measurement of CD and absorbance, no change in the CD parameter over this ammonium sulfate concentration range was observed. It is well established that aggregation, which is measured here by the absorbance at 329 nm, can cause distortions in ORD and CD spectra (Urry & Krivacic, 1970); indeed, when samples were prepared so that aggregation *would* take place, significantly lower molar el-

lipticity values were observed. Acid–urea–Triton gels indicated that there was no selective loss of a particular histone isotype due to the high concentrations of ammonium sulfate used in this study; thus, the observed optical properties represent the collective response of all histone polypeptides present in the sample.

The calculation of molar ellipticity is directly dependent upon the concentration of the protein sample being measured (Adler et al., 1973). The aggregation of histones will influence the accurate determination of protein concentration as the absorbance values at 277 and 329 nm will be affected due to the light scattering properties of the aggregated protein. Although there is a very small change in the extinction coefficient of histone octamers as a function of ammonium sulfate concentration (1%) in this range (Godfrey et al., 1990), it is not significant enough to alter the results.

The observations in this paper are at variance with an earlier study which described a complex pattern of molar ellipticity behavior for histones in the same range of ammonium sulfate concentrations, as well as a difference between histones in high ammonium sulfate buffers and those in 2 M sodium chloride (Park & Fasman, 1987), where solutions were clarified at 12 500 rpm for 30 min. Since unpublished results from the Johns Hopkins laboratory differed substantially from those reported by Park and Fasman (1987), extensive experiments on the solubility and aggregation behavior of the histone octamer were performed and are reported herein. The apparent changes in the circular dichroism signal of core histones in different ammonium sulfate buffers previously observed (Park & Fasman, 1987), although reproducible, are *not* due to structural changes induced by the solvents in question. Rather, they are due to aggregation which becomes more and more favorable as the ammonium sulfate concentration of the histone solution is increased. The high ammonium sulfate buffers examined approach the crystallization condition previously described (Burlingame et al., 1985), and it is expected that significant nucleation, aggregation, and precipitation will occur and manifest itself in the form of light scattering. Control of this behavioral aspect of the histone octamer yields reproducible results irrespective of the instrumentation used. It appears then that the earlier interpretation (Park & Fasman, 1987) of the apparent CD changes of histones dissolved in ammonium sulfate as conformational changes was in error. The histone octamer is indeed a conformationally flexible structure (Bidney & Reeck, 1977; Olins et al., 1977), but the suggestion that the histone octamer is so flexible as to undergo drastic changes in length in response to solvent perturbations, while appealing when offered as a means to reconcile the various models of the histone octamer, must be discounted given the results of this study. Clarification of histones in high-salt solutions by centrifugation at 30 000 rpm removed the discrepancy between the CD values obtained in 2 M NaCl and 2.0–2.5 M ammonium sulfate. Thus, the importance of scattering on circular dichroism measurements is once again stressed.

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