Newton, A. C., & Koshland, D. E., Jr. (1987) J. Biol. Chem. 262, 10185-10188.
Porter, D. C., Moy, G. W., & Vacquier, V. D. (1988) J. Biol. Chem. 263, 2750-2755.
Rosen, O. M., Rangel-Aldao, R., & Erlichman, J. (1977) Curr. Top. Cell. Regul. 12, 39-74.
Takai, Y., Nakaya, S., Inoue, M., Kishimoto, A., Nishiyama, K., Yamamura, H., & Nishizuki, Y. (1976) J. Biol. Chem. 251, 1481-1487.
Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., & Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397-402.
Taylor, S. S. (1987) BioEssays 7, 24-29.
Vardanis, A. (1980) J. Biol. Chem. 255, 1481-1487.
Vardanis, A. (1984) Biochem. Biophys. Res. Commun. 125, 947-953.
Walsh, D. A., Perkins, J. P., & Krebs, E. G. (1968) J. Biol. Chem. 243, 3763-3765.
Walter, U., Uno, I., Liu, A. Y.-C., & Greengard, P. (1977) J. Biol. Chem. 252, 6494-6500.

Salt Effects on Histone Subunit Interactions As Studied by Fluorescence Spectroscopy†

Catherine A. Royer,*,‡ Ruth M. Rusch,‡ and Suzanne F. Scarlata*,§

Department of Physics, Laboratory for Fluorescence Dynamics, University of Illinois at Urbana-Champaign, 1110 West Green Street, Urbana, Illinois 61801, and College of Medicine, Cornell University Medical College, 1300 York Avenue, New York, New York 10021

Received December 29, 1988; Revised Manuscript Received April 26, 1989

ABSTRACT: The salt concentration dependence of the aggregation properties of calf thymus and chicken erythrocyte histones has been investigated by using fluorescence spectroscopy. The isolated H2A/H2B and H3/H4 subunit preparations were labeled with 5-(dimethylamino)naphthalene-1-sulfonyl (dansyl). This long-lived fluorescence probe allows for the observation of rotations due to tumbling of the particle and thus is a probe for changes in the size of macromolecular assemblies. The fluorescence polarization and lifetime were measured as a function of salt concentration for these isolated preparations. Next, each labeled preparation was reconstituted with its unlabeled complement, and the salt concentration dependence of histone core octamer interactions was investigated in the same manner. Salt-induced core particle formation was observed by monitoring the dansyl-labeled dimers for both the calf thymus and chicken erythrocyte preparations. Evidence for subunit dissociation of the isolated H2A–H2B preparations was also found, as well as aggregation of the isolated H3/H4 subunits to at least dimers of tetramers. The calf thymus H3/H4 preparation was in aggregated form under all conditions studied, whereas the chicken erythrocyte H3/H4 only formed aggregates at high protein or salt concentrations. We have found evidence that the dimer can displace the tetramer from the higher order aggregate in order to form core particles. Such competition between the subunit interfaces in the histone system suggests that they may play a regulatory role in histone–DNA interactions.

Histones are small proteins that complex and condense eukaryotic chromosomal DNA [for a recent review, see McGhee and Felsenfeld (1980)]. Histones have been well characterized, and sequence studies have shown strong homology between species (Isenberg, 1979). Four major histones, H2A (14.5 kDa), H2B (13.8 kDa), H3 (15.3 kDa), and H4 (11.3 kDa), aggregate to an octamer that interacts with approximately 165 base pairs of DNA forming a nucleosome. The nucleosome consists of an $(H3)_2(H4)_2$ tetramer center flanked by two H2A–H2B[1] dimers in a tripartite arrangement (Richmond et al., 1984; Burlingame et al., 1985) with DNA looping twice around the outside of the protein core. A fifth histone, H1, binds to the DNA regions between the nucleosomes.

Due to the high content of basic amino acids, nucleosomes undergo a variety of structural changes with ionic strength [e.g., see Park and Fasman (1987)]. Below 200 mM salt, the two H2A–H2B dimers only weakly interact with the tetramer while remaining bound to DNA (Martinson et al., 1979), and at salt concentrations of approximately 2 M, DNA dissociates from the intact core octamer (Yager & van Holde, 1984). The loss of dimer–tetramer contacts has been postulated to account for the increased nuclease sensitivty of DNA in active genes and thus has been implicated in playing a role in nucleosome opening during transcription (Chao et al., 1979; Simpson & Stafford, 1983). In this work, we use fluorescence techniques to focus on histone–histone interactions and how these change as a function of ionic strength.

Much of our knowledge of nucleosome structure and possible dissociation mechanisms has been contributed by fluorescence studies. D'Anna and Isenberg (1974) and Libertini and Small (1982), using the intrinsic fluorescence of histone core particles, found evidence of a two-step H2A–H2B dimer dissociation from the octamer as the salt concentration

† This project was supported in part by BRSG S07 RR05396 to S.F.S. by the Biomedical Research Support Grant Programs Division of Research Resources, NIH. Additional support was provided in part by the University of Illinois at UC and by NIH Grants R29 GM399690 (C.A.R.) and P41 RR03155-01 (C.A.R. and R.M.R.).

* To whom correspondence should be addressed.
‡ University of Illinois at Urbana–Champaign.
§ Cornell University Medical College.

[1] Abbreviations: H2A–H2B, histone "dimer" subunits; H3/H4, histone "tetramer" subunits; DNS or dansyl, 5-(dimethylamino)-naphthalene-1-sulfonyl; CT, calf thymus; CE, chicken erythrocyte; SDS, sodium dodecyl sulfate; SAS, species-associated spectra; TBS, Tris-buffered saline; PBS, phosphate-buffered saline.

0006-2960/89/0428-6631$01.50/0

is lowered below 100 mM at pH 7.0. These fluorescence studies have been supported by other physical studies (Godfrey et al., 1980; Benedict et al., 1984). Chung and Lewis (1986) covalently labeled H4 in the H3/H4 interface region and concluded from energy-transfer measurements that from ionic strengths of 100 to 600 mM there is a general loosening of the particle and that contacts within the $(H3)_2(H4)_2$ tetramer become disrupted.

In this study, the fluorescence properties of histones labeled with the probe 5-(dimethylamino)naphthalene-1-sulfonate (dansyl) will be monitored. The advantage of using dansyl-labeled protein is that the long probe lifetime allows us to monitor changes in polarization that are primarily a function of the aggregation state of the protein. The average molecular volume calculated from Perrin's law (Perrin, 1926) results from a combination of local dansyl motions and global protein tumbling. In the absence of local motions, the measured molecular volume in milliliters per mole is approximately equal to the molecular weight. The values thus measured give a lower limit to the molecular weight. Dansyl covalently labels amine groups, and because the histones are rich in lysine residues, many labeling sites are possible. The labeling ratio was kept below one label per subunit to minimize any perturbing effects. Since we are interested in observing the general effects of ionic strength on the subunit affinities of our complexes, knowledge of the exact labeling sites is not necessary.

We have studied the isolated H2A–H2B as well as the H3/H4 subunits from both calf thymus and chicken erythrocyte chromatin individually labeled with dansyl chloride. Since we are studying aggregation states, the H3 and H4 subunit solutions are referred to as H3/H4. The fluorescence polarization and lifetime of these preparations were measured as a function of sodium chloride concentration between 200 mM and 2 M. Each labeled solution was then reconstituted with its unlabeled complement (e.g., unlabeled H3/H4 was added to dansyl-labeled H2A–H2B and vice versa), and the salt concentration effect upon the subunit interactions in these complexes was explored. In this manner, we have made a qualitative characterization of the modulation of histone–histone interactions by ionic strength. The following paper (Scarlata et al., 1989) describes the quantitative determination of the various subunit affinities by combining the fluorescence techniques with high-pressure perturbations.

Materials and Methods

Sample Preparation. Calf thymus histones were purchased from Sigma Chemicals, St. Louis, MO. The powder was solubilized to high concentrations in aqueous salt solutions by dropping the pH to 4, slowly adding salt, and then increasing the pH. SDS gel electrophoresis revealed that the mixture consisted of approximately equal amounts of the five major histones with trace amounts of two low-mobility proteins. Calf thymus H2A and H2B were separated from the $(H3)_2(H4)_2$ tetramer by gel exclusion chromatography on a Sephadex G-100 Superfine column at pH 7.0 and at 200 mM NaCl, according to Callaway et al. (1984). When SDS–polyacrylamide gel electrophoresis was used, the first eluting peak was found to contain approximately 85% H3 and H4 and 15% H1, while the second eluting peak contained exclusively H2A–H2B.

Chicken erythrocyte chromatin was prepared, and the individual histone subunits were purified following the method of Simon and Felsenfeld (1979), with modifications (Dr. Jon Widom, personal communication). Chicken erythrocytes were isolated from chicken blood by repeated centrifugation at 700*g* and resuspension in PBS. Digestion was performed in TBS at pH 8 in 1 μM $CaCl_2$ for 30 min and the chromatin isolated by centrifugation. Core histone particles were prepared by elution from a hydroxyapatite column (Simon & Felsenfeld, 1979). After extensive washing at 0.63 M NaCl, a buffer change to 2 M NaCl resulted in elution of pure core. In order to obtain pure H2A–H2B and H3/H4 fractions, a linear salt gradient was run from 0.93 to 1.2 M NaCl. SDS–polyacrylamide gel electrophoresis of the purified H2A–H2B and the H3/H4, as well as the purified core particle, showed no contaminating species in any sample.

The histones were labeled with the fluorescent probe, 5-(dimethylamino)naphthalene-1-sulfonate (dansyl), as follows. Approximately 2 mL of the histone mixture or histone fraction at concentrations between 0.5 and 2 mg/mL in 100 mM potassium phosphate buffer, pH 8, was mixed with 20 μL of 20 mM dansyl chloride in dimethylformamide. The reaction was allowed to proceed for 10 min at room temperature. The solution was either dialyzed 6 times against 1 L of 50 mM Tris, pH 7, at 5 °C, or separated from free dye over a Sephadex G-25 Superfine column and dialyzed once. The last dialysis buffer showed no evidence of free dansyl in either the absorption or the emission spectra.

Dansyl concentration was determined by using a molar extinction coefficient of 4500 M^{-1} cm^{-1} at 340 nm. Protein concentrations of the unlabeled protein solution were also measured by absorption at 275 nm using extinction coefficients of 4000 and 5000 cm^{-1} M^{-1} for the dimer and tetramer, respectively (D'Anna & Isenberg, 1974) and 3600 cm^{-1} M^{-1} for dansyl. Labeling ratios, determined by assuming approximate linear additivity of absorption coefficients of the protein and the dye, were always below one dansyl per histone monomer. All measurements were conducted at 22 °C in 50 mM Tris, pH 7.0.

Gel exclusion chromatography was performed using a Sephadex G-100 column calibrated with blue dextran, ovalbumin, and RNaseA. The elution buffer was 20 mM in Tris and either 200 mM or 2 M in NaCl, pH 7.0. In both cases, 40 μM in H3/H4 monomer was loaded in a volume of 1.0 mL. Flow rates were 0.25 mL/mol.

Instrumentation. Absorption measurements were made on a Perkin-Elmer Lambda-V (Norwalk, CT). Fluorescence emission and polarization were carried out on a Greg-PC (I.S.S. Inc., Champaign, IL) using the 325-nm line of a Liconix HeCd continuous-wave laser or a Xenon arc lamp at 340 nm for excitation and an emission wavelength of 500 nm for polarization. Fluorescence lifetimes were measured by using the multifrequency phase-modulation fluorometer described by Gratton and Limkeman (1983), using the 325-nm line of a HeCd laser and a KV370 cuton filter in emission. For each sample, 8–12 frequencies, ranging from 1 to 100 MHz, were measured using magic-angle excitation.

Measurements. All measurements were done at 20 °C. Except for the dilution studies, the average error of the polarization values did not exceed ±0.004, while the errors in phase angle and modulation ratio were below ±0.200° and ±0.004, respectively. Polarization values, under identical conditions for different labeling preparations, were the same within 5%.

Data Analysis. Average molecular volumes ($\langle V_m \rangle$) were calculated from the raw polarization values (p) and average lifetimes ($\langle \tau \rangle$) by using the Perrin equation:

$$\frac{1/p_0 - 1/3}{1/p - 1/3} - 1 = RT\langle \tau \rangle / \eta V_m$$

where p_0 is the polarization in the absence of rotational motion,

Table I: Dansylleucine Fluorescence Properties

solvent	$\bar{\nu}g$ (cm^{-1})	$\langle\tau\rangle$ (ns)
H_2O	17718	3.4
2 M NaCl	17449	3.2
glycerol	18315	9.0
BuOH	19183	14.2

which for dansyl is 0.4 (Weber, 1951), R is the gas constant, T is the absolute temperature, and η is the viscosity of the solvent. Viscosities for the various salt solutions were taken from the *CRC Handbook of Chemistry and Physics* (1986–1987).

Lifetime Analysis. Multifrequency phase and modulation data sets were analyzed simultaneously by using the program "Globals Unlimited" described by Beechem and Gratton (1988). Analysis of the mutlifrequency phase and modulation data of every sample at all salt concentrations always gave the best fit to a model assuming three distinct and well-separated lifetime components. The recovered lifetime values for the various samples will be discussed in the following sections.

Values of χ^2 increased 5–30-fold when fits were attempted using either double-exponential or single- or double-distribution models. Also, single-curve analyses assuming triple distributions of lifetimes always gave the smallest allowable width for each component. Therefore, we concluded that at least three discrete classes of dansyl residues, long-, medium-, and short-lived, best describe the fluorescence decay. The lifetime data from all salt concentrations for each protein species best fit a "linked" model (Beechem et al., 1983; Knutson et al., 1983) in which the addition of salt is assumed to have no effect on the values of the individual lifetimes, but rather their preexponential weighing factors (SAS, or species-associated spectra). Although the decay kinetics of such heterogeneous systems are surely highly complex, this physical model assumes that the addition of salt causes conformational changes or modifications in subunit interactions that in turn cause changes in the relative population of the three different classes of decays. Rigorous confidence interval tests involving a complete minimization at each tested value of a given parameter were performed on the recovered lifetime values for the protein preparations, and worst case errors confirm that the dansyl–histone decays indeed correspond to three distinct species. Error bars were obtained for the recovered values by determining the lowest and highest SAS values for which the χ^2 falls below the 67% confidence level (1 standard deviation of the Gaussian distribution of error). Errors on recovered SAS values were approximately ±0.05 and were comparable for all protein solutions except calf thymus H2A–H2B–DNS in the presence of H3/H4. This sample displayed larger errors due to low light levels and high gain, but similar smooth trends in the SAS values were observed. Unlinking the lifetimes resulted in essentially no difference in the recovered parameter or the χ^2 values. Lifetimes of dansylleucine in water, 2 M aqueous NaCl, glycerol, and butanol are given in Table I. It can be seen that although salt has no effect on the dansyl lifetime, the more polar the solvent, the faster the decay.

Results

Salt Titrations of Calf Thymus H2A–H2B–DNS Complexes

Polarization Results. The polarization and, thus, the molecular volume of the dansyl-labeled calf thymus (CT) H2A–H2B dimer from 8 to 4 μM remained constant between 10 mM and 2 M salt (Figure 1a and Table I). Upon dilution from 8 to 2 μM dimer into a solution containing 2 μM H3/H4,

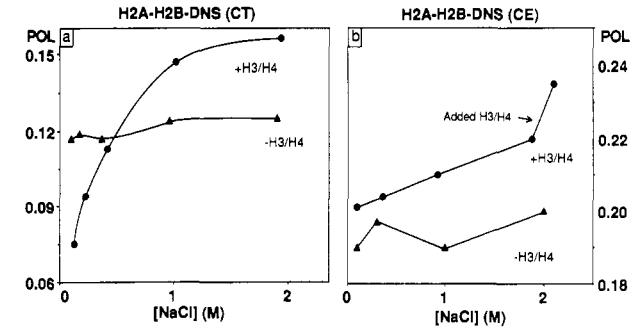

FIGURE 1: Salt titration of the dansylated histone dimers. (a) CT denotes calf thymus dimer and (b) CE chicken erythrocyte. In both cases, studies were done in the absence (–H3/H4) and in the presence (+H3/H4) of the unlabeled H3/H4 complements. Starting concentrations were 8 μM for the CT DNS–H2A–H2B alone and 2 μM in the presence of unlabeled H3/H4. The CE DNS dimer had an initial concentration of 20 and 6 μM in the presence of unlabeled H3/H4. All solutions were diluted by a factor of 2 by the salt titration.

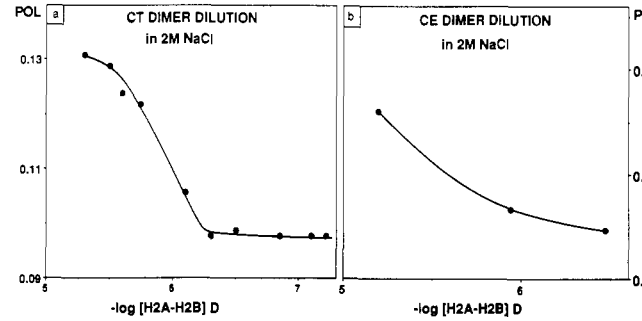

FIGURE 2: Dilution polarization profiles in 2 M NaCl of the dansyl-labeled H2A–H2B solutions (a) from calf thymus (CT) and (b) from chicken erythrocyte (CE).

the polarization of the dansyl-labeled H2A–H2B decreased from 0.12 to 0.075, corresponding to a 2-fold decrease in the molecular volume (Table I). Since very little interaction should occur between H2A–H2B and H3/H4 at these salt concentrations, we suspected that the decrease in polarization upon dilution was due to dissociation of the calf thymus H2A–H2B dimer or of a higher order aggregate. We performed a dilution experiment on calf thymus H2A–H2B–DNS in 2 M NaCl. The polarization values decreased 0.04 polarization unit between 8 and 2 μM in dimer (Figure 2), although the error became rather high, ±0.01, below 1 μM in dimer. Such a decrease in polarization upon protein dilution is entirely consistent with protein subunit dissociation.

Figure 1a also shows the polarization of the dansyl-labeled H2A–H2B in the presence of unlabeled H3/H4 as a function of increasing NaCl concentration. The polarization exhibits a large increase over this salt range, whereas no change was observed in the absence of H3/H4. Such a result is consistent with core particle formation. The average lifetime increased only slightly from 11.4 to 12.6 ns. From these values, the change in the average rotational volume was calculated to increase from 6200 to 15900 mL/mol (Table I). It should be noted that these values for the molecular volumes are averages calculated from the total depolarization, which includes contributions from both the local rotations of the dansyl label and the global rotations of the protein. Thus, the 2.5-fold salt-induced increase in the polarization of the labeled dimer when tetramer is present is consistent with approximately 50% formation of core particle.

Lifetime Results. The frequency response of the dansyl-labeled calf thymus H2A–H2B, both in the absence and in the presence of unlabeled H3/H4 as a function of salt con-

Table II: H2A–H2B–DNS Polarization Results

source	$[H2A–H2B]_D$[a] (μM)	$[H3/H4]_T$[a] (μM)	[NaCl] (M)	pol	$\langle\tau\rangle$ (ns)	$\langle V\rangle_m$ (mL/mol)
CT	8.0	0.0	0.01	0.117	11.6	11 400
CT	4.0	0.0	2.00	0.125	11.9	11 400
CT	2.0	7.7	0.10	0.075	11.4	6 200
CT	1.0	3.8	2.03	0.156	12.6	15 900
CE	20.0	0.0	0.10	0.190	11.3	17 700
CE	10.0	0.0	2.00	0.200	12.1	18 600
CE	6.0	6.0	0.10	0.201	11.7	27 700
CE	3.0	3.0	2.10	0.235	12.3	33 600

[a] Subscripts D and T refer to the protein concentration expressed as dimer and tetramer, respectively.

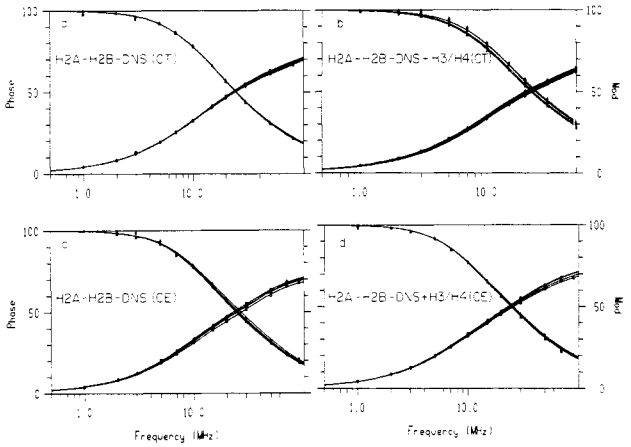

FIGURE 3: Frequency response of the dansyl-labeled histone dimers as a function of salt concentration. (a) Calf thymus dansyl-labeled dimer; (b) calf thymus dansyl-labeled dimer in the presence of unlabeled H3/H4; (c) chicken erythrocyte dansyl-labeled dimer; (d) chicken erythrocyte dansyl-labeled dimer in the presence of unlabeled H3/H4. Concentrations are identical with those in Figure 1.

centration, is shown in Figure 3a,b. While it can be seen that the addition of salt has very little effect on the H2A–H2B–DNS decay in the absence of unlabeled H3/H4, there is a shift to lower frequencies of the phase angle and demodulation as a function of salt concentration when H3/H4 is present. This is simply a result of a decrease in the average decay rate (Table I). While lifetime values from the linked analysis (see Materials and Methods) for the three components were the same in the absence and in the presence of the unlabeled H3/H4 histones within the confidence limits on the recovered parameters (Table II), the fractional contribution of the long component increased by approximately 10% over this salt concentration range when H3/H4 was present.

Salt Titrations of Chicken Erythrocyte H2A–H2B–DNS Complexes

Polarization Results. In order verify the reproducibility of the data obtained with calf thymus histones, the individual histones were purified from chicken erythrocytes and labeled as in the case of the calf thymus preparation. The polarizations and lifetimes were measured for the different labeled histones and their reconstituted core particles.

The polarization of 1.2 μM H2A–H2B–DNS dimer from chicken erythrocytes (CE) was measured as a function of a salt concentration. As shown in Figure 1b, no significant increase in the polarization occurs between 100 mM and 2 M NaCl. The small changes observed in polarization of the dansyl chicken erythrocyte dimer over this salt range are interpreted as being due to salt-induced local conformational changes in the tertiary structure. From the average lifetime and polarization values, the molecular volumes were calculated to be nearly constant (17 700–18 600 mL/mol) (Table II). These values are close to those expected for a dimer of molecular weight 28.3K. We interpret the lower values for the calf thymus dimer to be a result of the 2-fold higher labeling ratio (1/monomer as opposed to 1/dimer for the CE H2A–H2B–DNS) and a greater degree of local rotation for the dansyl residues in the calf thymus dimer. Dilution profiles of the polarization for the chicken erythrocyte dansyl dimer again showed some evidence for higher order aggregate dissociation although the effect was much smaller than for the calf thymus preparation (Figure 2b).

Table III: Lifetime Values for the Dansyl Solutions

source	solution	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	χ^2
CT	H2A–H2B–DNS	15.9 ± 0.3	7.2 ± 1.0	1.9	2.2
	H2A–H2B–DNS + H3/H4	16.2 ± 0.5	8.1 ± 2.0	2.0	6.9
CT	H3/H4–DNS	18.8 ± 0.3	8.2 ± 1.0	2.0	1.6
	H3/H4–DNS + H2A–H2B	19.2 ± 0.4	8.5 ± 1.0	2.1	0.9
CE	H2A–H2B–DNS	15.5 ± 1.0	4.9 ± 1.0	0.2	3.4
	H2A–H2B–DNS + H3/H4	16.9 ± 2.0	6.0 ± 2.0	1.2	1.7
CE	H3/H4–DNS	19.7 ± 2.0	8.5 ± 2.0	1.6	1.7
	H3/H4–DNS + H2A–H2B	18.8 ± 2.0	8.7 ± 2.0	2.1	3.1

In the presence of unlabeled H3/H4 (Figure 1b), there is an increase in the polarization at 100 mM salt as compared to that of the dimer alone. This corresponds to a molecular volume of 27 700 mL/mol and indicates some interaction between the H2A–H2B–DNS and the H3/H4 at that salt concentration. As the salt concentration increases, the polarization steadily rises to a value of 0.22 at 2 M NaCl. When excess H3/H4 is added, keeping the salt concentration constant at 2 M, the polarization increases further to 0.235 (molecular volume of 33 600 mL/mol, Table II), against consistent with core particle formation. We have also observed an increase of approximately 0.05 polarization unit in the H2A–H2B–DNS polarization at 2 M salt upon increasing the total core particle protein concentration (labeled dimer and unlabeled tetramer) between 0.1 and 10 μM. These results are also consistent with core histone octamer formation.

Lifetime Results. The frequency response of the chicken erythrocyte H2A–H2B–DNS fluorescence was measured as a function of salt concentration in the absence and in the presence of unlabeled H3/H4, and the results were analyzed globally as for the calf thymus preparations. The results are shown in Figure 3c,d. While the average lifetime values shown in Table II were shorter than those in the calf thymus preparations, the value of the long-lived component was strikingly similar, presumably due to the relatively high degree of homology between histones of different origins (Isenberg, 1979). In absence of H3/H4, there is a shift of the phase and modulation plots to lower frequency as salt is added (Figure 3c). The average lifetime increased slightly from 11.3 to 12.1 ns (Table II). A slight salt effect is also observed for the frequency response of the chicken erythrocyte H2A–H2B–DNS in the presence of H3/H4 (Figure 3d). The average lifetime increases from 11.7 to 12.3 ns (Table II). The recovered

Table IV: H3/H4–DNS Polarization Results

source	$[H3/H4]_T$[a] (μM)	$[H2A–H2B]_D$[a] (μM)	[NaCl] (M)	pol	$\langle\tau\rangle$ (ns)	$\langle V\rangle_m$ (mL/mol)
CT	0.5	0.0	0.05	0.255	14.5	60 000
CT	0.25	0.0	2.00	0.246	14.5	46 000
CT	7.7	7.7	0.03	0.249	14.5	56 400
CT	3.8	3.8	2.00	0.246	14.9	46 900
CE	3.6	0.0	0.20	0.261	10.6	46 200
CE	1.8	0.0	2.00	0.292	10.8	57 500
CE	3.6	0.0	2.00	0.292	11.0	57 500
CE	1.8	4.3	2.00	0.292	10.9	57 800

[a] Subscripts D and T refer to the protein concentration expressed as dimer and tetramer, respectively.

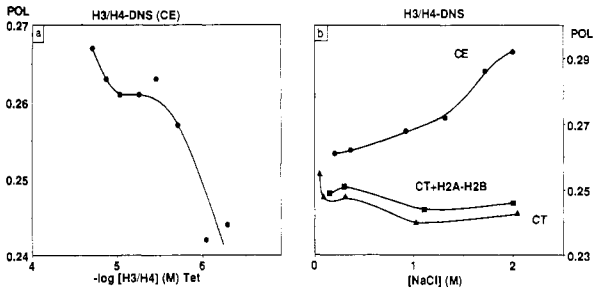

FIGURE 4: Polarization studies of dansyl-labeled histone H3/H4 solutions. CE denotes chicken erythrocyte histones, and CT denotes calf thymus histones: (a) dilution polarization profile of dansyl-labeled chicken erythrocyte H3/H4 preparation; (b) salt polarization titration of dansyl-labeled chicken erythrocyte H3/H4 (CE) (3.6 μM in tetramer), dansyl-labeled calf thymus H3/H4 (CT), and dansyl-labeled calf thymus H3/H4 (CT) 0.5 and 7.7 μM in the absence and in the presence of unlabeled dimer (+H2A–H2B), respectively. Solutions were diluted by a factor of 2 upon titration with NaCl.

lifetime values for the three components (Table III) are slightly higher when H3/H4 is present.

Salt Titrations of Calf Thymus H3/H4–DNS Complexes

Polarization Results. A salt titration of the calf thymus H3/H4–DNS complex showed little change in polarization over the entire ionic strength range (Figure 4b). However, when changes in solvent viscosity and lifetime are considered, the molecular volume decreased from 60 000 to 46 000 mL/mol over this range (Table III). Since there was no clear trend and the changes occurred at very low salt, we interpret these as being due to changes in the degree of local mobility of the dansyl residues upon salt-induced conformational differences in the protein. When unlabeled H2A–H2B was added to the solution, the polarization values were only slightly higher. The values calculated for the average molecular volumes (approximately 55 000 mL/mol) were slightly higher for the H3/H4 tetramer of approximately 53 000 daltons, indicating possible higher order aggregation. Thus, we ran the labeled and unlabeled H3/H4 samples at 1–10 μM in tetramer over a Sephadex G-100 column. In both cases, the protein eluted in the void volume both in 2 M and in 200 mM NaCl, indicating that the size of the calf thymus H3/H4 complex was larger than tetramer under all conditions tested.

Lifetime Results. The average lifetime (Table IV) and the three individual lifetime values for the dansyl-labeled calf thymus H3/H4 (Table III) were found to be longer than those of both the calf thymus and chicken erythrocyte dimers. In particular, the value for the long-lived component was found to be between 18.8 and 19.7 ns, compared to 15.5–16.5 ns for the dansyl-labeled dimers. Salt had little effect on the calf thymus H3/H4–DNS fluorescence lifetime. The average lifetime showed no change in the absence of H2A–H2B and only a small increase in its presence. The changes in the SAS values with increasing NaCl concentrations were within the rigorous 67% confidence intervals (Beechem & Gratton, 1987) for the recovered values, and the raw data showed no obvious changes.

Salt Titration of the Chicken Erythrocyte H3/H4–DNS Complexes

Polarization Results. Figure 4a shows the dilution profile of the chicken erythrocyte dansyl-labeled H3/H4 complex. A slight decrease can be seen in the polarization as the complex is diluted from 25 to 8 μM in tetramer, followed by a plateau region, and finally a decrease again below 2 μM in tetramer. Figure 4b shows the salt dependence of the polarization of a 3.6 μM H3/H4 (tetramer) solution. This increase in the polarization, with a corresponding increase in the molecular volume from 46 200 to 57 500 mL/mol (Table IV), was interpreted as due to salt-induced higher order aggregation of the H3/H4–DNS solution. In order to confirm this hypothesis, gel exclusion chromatography was carried out on unlabeled chicken erythrocyte H3/H4. At 200 mM NaCl, 1 mL of 10 μM H3/H4 (tetramer) elutes essentially as a tetramer of molecular weight near 40 000. When the column was run at 2 M NaCl, however, the protein eluted in the void volume. Thus, while the calf thymus H3/H4 was an aggregate of tetramers (at least dimer of tetramer) under all salt concentrations tested, the chicken erythrocyte H3/H4 only formed higher order aggregates at high salt concentrations. The value for the chicken erythrocyte H3/H4–DNS polarization in the presence of unlabeled H2A–H2B at high salt concentration was the same as that measured in the absence of dimer.

Lifetime Results. The frequency response of the chicken erythrocyte H3/H4–DNS fluorescence was measured as a function of salt concentration, and the data were analyzed globally as above. As in the case of the labeled dimer, the recovered lifetime values were very similar to those found for the calf thymus preparations (Table III). The fractional concentration of the long-lived component was smaller, resulting in a lower average lifetime (Table IV). Again, very little salt dependence of the frequency response of the dansyl fluorescence was evident. Thus, the calf thymus and chicken erythrocyte H3/H4 environments appear to be very similar. No change in the lifetime or polarization of this complex was observed upon addition of dimer at 2 M salt. However, the labeled dimer polarization results show core particle formation under the same conditions, and, thus, two dimer molecules must replace one tetramer H3/H4 aggregate to form the core particle octamer. Since it is approximately the same size as the H3/H4 octamer, the average molecular volume for the H3/H4–DNS shows no change upon displacement of the tetramer by the dimers.

DISCUSSION

The work presented here focuses on the use of fluorescence spectroscopy to investigate the effects of ionic strength upon histone subunit interactions. Labeling with a long-lived fluorescent probe, dansyl, allowed us to follow the size of the histone complexes as a function of protein and salt concen-

trations. Although the decay and rotational kinetics of fluorescent probes in biological molecules may be extremely complex, in the absence of any large lifetime effects, changes in the steady-state polarization values which occur as a function of protein concentration have their origin in aggregation phenomena. We have observed salt and protein concentration-dependent changes in polarization and not lifetime only under conditions where aggregation between the H2A–H2B and H3/H4 histone subunits was possible. We have also verified that these changes are not simply due to changes in ionic strength.

The addition of unlabeled H3/H4 to the dansyl-labeled dimer resulted in a very large salt-induced increase in the fluorescence polarization for both the calf thymus and chicken erythrocyte dimer preparations, whereas no change was observed in the absence of H3/H4, indicating, indeed, that high salt concentration promotes core particle formation. Since the increase in dansyl polarization does not occur for the labeled dimers in the absence of H3/H4, we conclude that dimer binding to tetramer is responsible for the observed increase in rotational volume. In the case of chicken erythrocyte (CE) labeled dimer, a small salt-induced polarization increase was observed when H3/H4 was present, and the addition of 2.5 times more unlabeled H3/H4 (1.8–4.6 μM) caused an additional increase in polarization, corresponding to further core particle formation. The total change in the average molecular volume, $\langle V_m \rangle$, for 1.8 μM dimer in the presence of 4.6 μM H3/H4 as a function of increasing salt concentration was ~18000 to ~33000 mL/mol. Formation of 100% core would correspond to 72000 mL/mol or a 4-fold increase in $\langle V_m \rangle$. Thus, we observed approximately 30% aggregation to core octamer. The concentration dependence of CE core octamer formation also indicateds that association occurs in the micromolar range. This is in agreement with the previous work of Godfrey and co-workers (Godfrey et al., 1980).

In the case of the calf thymus (CT) labeled dimer, upon addition of salt in the presence of unlabeled H3/H4, we measured a 2.5-fold increase in molecular volume. This corresponds to 52% of the total change for core particle formation. Thus, the average K_d for the dimer–tetramer interaction appears also to be in the micromolar range. Using equilibrium sedimentation, Bendict et al. (1984) found a K_d of approximately 1.2 μM for the dimer–tetramer interaction.

The polarization and lifetime data for the labeled H3/H4 preparations revealed that these histones aggregate to at least dimers of tetramers under certain conditions of protein and salt concentration. In the case of the calf thymus H3/H4, the protein was at least octameric under all conditions studied in this work. D'Anna and Isenberg (1974) observed aggregation of H3 and H4 subunits, but not the H3/H4 mixture. This is most likely due to the fact that with a relatively short fluorescence lifetime (1–4 ns), the tyrosyl residues which they were observing were not sensitive to the formation of large aggregates. In our case, the dansyl lifetime is much longer (10–15 ns) and well-suited to the study of subunit equilibria in macromolecular complexes.

The chicken erythrocyte H3/H4 preparation was tetrameric below 5 μM and in 200 mM salt. However, as the salt concentration was raised to 2 M in NaCl, the chicken erythrocyte H3/H4 also formed higher order aggregates, as evidenced by dansyl polarization and gel exclusion chromatography. This behavior is presumably due to charge shielding. At 2 M salt, the polarization showed no change upon addition of unlabeled dimer, although an increase in polarization of the labeled dimer was observed in the presence of unlabled tetramer under the same conditions. This is consistent with the displacement of H3/H4 from the H3/H4 octamer by two H2A–H2B dimers to form core particle octamers. Of course, since the total size of the particle does not change appreciably when this occurs (both particles would be octamers and should have similar rotational volumes), no significant change in the H3/H4–DNS polarization was observed. Such a displacement implies that although the affinity between the dimer and tetramer is higher than that of the tetramer for itself, a competition exists between core particle formation and H3/H4 aggregation under conditions of high salt concentration.

The regulation of histone dimer–tetramer interactions through relatively small changes in the ionic strength of the local environment could affect the affinity of the core particle for DNA. Since dimer–tetramer and tetramer–tetramer contacts are extremely sensitive to changes in ionic strength, alterations in subunit affinities could influence core particle formation and nucleosome unfolding. Benedict et al. (1984) also suggested from calorimetric studies that the subunit interfaces could be involved in regulating histone–DNA interactions. Thus, the different subunit interfaces in the histone core particle could play an important role in the energetics of processes such as transcription and chromosome packing.

ACKNOWLEDGMENTS

We thank Drs. Mark Gardener and Jon Widom for assistance and advice in the chicken erythrocyte histone preparations. The experiments and analyses of the data produced were performed at the Laboratory for Fluorescence Dynamics (LFD) at the University of Illinois at Urbana–Champaign (UIUC). The LFD is supported jointly by the Division of Research Resources of the National Institutes of Health (RR03155) and by UIUC. We are also grateful to Julie Butzow for the preparation of the manuscript and the computer-generated figures.

REFERENCES

Beechem, J., & Gratton, E. (1988) *Proc. SPIE—Int. Soc. Opt. Eng. 909*, 70–81.

Beechem, J. M., Knutson, J. R., Ross, J. B. A., Turner, B. W., & Brandt, L. (1983) *Biochemistry 22*, 6054–6058.

Benedict, R. C., Moudrianakis, E. N., & Ackers, G. A. (1984) *Biochemistry 23*, 1214–1218.

Burlingame, R. W. Love, W. E., Wang, B. C., Hamlin, R., Xuong, N.-H., & Moudrianakis, E. N. (1985) *Science 228*, 546–553.

Callaway, J. E., Ho, Y., & DeLange, R. J. (1985) *Biochemistry 24*, 2692–2697.

Chao, M. V., Gralla, J., & Martinson, H. G. (1979) *Biochemistry 18*, 1068–1074.

Chung, D. G., & Lewis, P. N. (1986) *Biochemistry 25*, 5026–5042.

CRC Handbook of Chemistry and Physics (1986–1987) 56th ed., pp 251–258, CRC Press, Boca Raton, FL.

D'Anna, J. A., & Isenberg, I. (1974) *Biochemistry 13*, 4987–4992.

Eichbuch, T. H., & Moudrianakis, E. N. (1978) *Biochemistry 17*, 4955–4964.

Godfrey, J. E., Eichbuch, T. H., & Moudrianakis, E. N. (1980) *Biochemistry 19*, 1339–1346.

Gratton, E., & Limkeman, M. (1983) *Biophys. J. 44*, 315–323.

Isenberg, I. (1979) *Annu. Rev. Biochem. 48*, 159–191.

Knutson, J. R., Beechem, J. M., & Brand, L. (1983) *Chem. Phys. Lett. 102*, 501–507.

Libertini, L. J., & Small, E. W. (1982) *Biochemistry 21*, 3327–3334.

Martinson, H. G., True, R. J., & Burch (1979) *Biochemistry 18*, 1082–1088.
McGhee, J. D., & Felsenfeld, G. (1980) *Ann. Rev. Biochem. 49*, 1115–1156.
Park, K., & Fasman, G. D. (1987) *Biochemistry 26*, 8042–8045.
Perin, F. (1926) *J. Phys. Radium 1*, 390–401.
Richmond, T. J., Finch, J. T., Rushton, B., Rhodes, D., & Klug, A. (1984) *Nature 311*, 532–538.
Scarlata, S. F., Ropp, T., & Royer, C. A. (1989) *Biochemistry* (following paper in this issue).
Simon, R. H., & Felsenfeld, G. (1979) *Nucleic Acids Res. 6*, 689–696.
Simpson, R. T., & Stafford, D. W. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 51–55.
Weber, G. (1951) *Biochem. J. 51*, 155–164.
Yager, T. D., & Van Holde, K. E. (1984) *J. Biol. Chem. 259*, 4212–4222.

Histone Subunit Interactions As Investigated by High Pressure†

Suzanne F. Scarlata,*,‡ Traci Ropp,§ and Catherine A. Royer*,§

College of Medicine, Cornell University Medical College, 1300 York Avenue, New York, New York 10021, and Department of Physics, Laboratory for Fluorescence Dynamics, University of Illinois at Urbana-Champaign, 1110 West Green Street, Urbana, Illinois 61801

Received December 29, 1988; Revised Manuscript Received April 26, 1989

ABSTRACT: High-pressure fluorescence polarization was used to investigate subunit interactions of the histone H2A–H2B dimer and the H3/H4 tetramer isolated from calf thymus (CT) and chicken erythrocyte (CE) chromatin. The proteins were individually labeled with the fluorescent probe 5-(dimethylamino)-naphthalene-1-sulfonate (dansyl or DNS), and the fluorescence polarization was measured as a function of pressure. The long fluorescence lifetime of the probe allows for the observation of global rotations of the protein, the rate of which is dependent upon the aggregation state. From the pressure dependence of the dansyl polarization, the K_d of H2A–H2B dissociation of the CE dimer was found to be approximately 1×10^{-7} M at 2.0 M NaCl. Lowering the salt concentration to 200 mM slightly stabilized the protein to 6×10^{-8} M. Our data indicate a small negative volume change for the dissociation of the core particle octamer. The $(H3)_2(H4)_2$ tetramer, as was shown in the previous paper (Royer et al., 1989), also formed predominantly dimers of tetramers at higher protein or salt concentrations. In the study presented here, we found the dissociation constant for the H3/H4 octamer to dimer transition to be 1×10^{-21} M^3 ($C_{1/2} = 4 \times 10^{-8}$ M) at 2 M NaCl for the CT preparation. Decreasing the salt concentration to 200 mM reduced the stability of the CT H3/H4 octamer to 9×10^{-21} M^3 ($C_{1/2} = 8 \times 10^{-8}$ M). The dimer of the CE tetramer also dissociated upon application of pressure in 2 M salt. The K_d for this equilibrium was found to be 1.4×10^{-9} M. At lower salt and protein concentrations, CE $(H3)_2(H4)_2$ was predominantly tetramer, and we found the tetramer to dimer dissociation constant to be 1.5×10^{-8} M under these conditions.

Chromatin is packaged into a dense protein–DNA complex consisting of small units called nucleosomes. In these structures, the DNA is wrapped around a $(H2A–H2B)_2[(H3)_2(H4)_2]$[1] histone octamer [for a general review, see McGhee and Felsenfeld (1980)]. The mechanism of nucleosome assembly and unfolding during transcription is not yet known. The purpose of this study is to better understand these processes by determining the energetics of histone core interactions and how these may be effected by changes in the ionic strength. In the preceding paper (Royer et al., 1989), we focused on the formation of the core histone octamer by measuring the changes in fluorescence polarization and lifetime of histones labeled with 5-(dimethylamino)naphthalene-1-sulfonyl (dansyl or DNS). This method allows for the observation of changes in polarization which correspond to changes in the aggregation state of the protein. In the study presented here, the dissociation of various dansyl–histone complexes was measured by observing the dansyl polarization as a function of hydrostatic pressure. From LeChatlier's principle, increasing the pressure on a system in equilibrium will shift the equilibrium toward the side which occupies the least volume. Dissociation of a globular oligomeric protein into its subunits occurs with a reduction in volume due to more efficient packing of water between the subunits, replacement of nonpolar interactions with tighter polar interactions, and an increase in electrostriction between water and charges that result from the breaking of intersubunit salt bridges (Heremans, 1982; Weber & Drickamer, 1983). Thus, applying pressure to solutions of oligomeric proteins generally results in their dissociation. If the protein is labeled with dansyl, a decrease in polarization with pressure occurs due to a decrease in rotational volume upon subunit dissociation. From the pressure versus polarization curve, we can calculate the dissociation constant, K_d,

† This project was supported in part by National Institutes of Health Grant GM39924 (S.F.S.), by the University of Illinois at UC, and by NIH Grants R29 GM399690 (C.A.R.) and P41 RR03155-01 (C.A.R. and T.R.).

* To whom correspondence should be addressed.
‡ Cornell University Medical College.
§ University of Illinois at Urbana–Champaign.

[1] Abbreviations: H2A–H2B, histone "dimer" subunits; H3/H4, histone "tetramer" subunits; DNS or dansyl, 5-(dimethylamino)-naphthalene-1-sulfonyl; CT, calf thymus; CE, chicken erythrocyte; SDS, sodium dodecyl sulfate; SAS, species-associated spectra; TBS, Tris-buffered saline; PBS, phosphate-buffered saline.

0006-2960/89/0428-6637$01.50/0