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Histone Subunit Interactions As Investigated by High Pressure†

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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,

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[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.

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Table I: Dissociation Parameters for Dansyl-Labeled Histone Dimers

source	[H2A–H2B] (μM)	[NaCl] (M)	K_d (M)	$C_{1/2}$ (M)	ΔV_a[a] (mL/mol)	$\langle V\rangle_m$[a](atm) (mL/mol)	$\langle V\rangle_m$[a](2 kbar) (mL/mol)
CE	2	0.2	6×10^{-8}	6×10^{-8}	90 ± 4	17700	7200
CE	2	2.0	1×10^{-7}	1×10^{-7}	75 ± 5	18600	13000

[a] ΔV refers to the thermodynamic volume change of the reaction, and $\langle V\rangle_m$ refers to the hydrodynamic volume extracted from the fluorescence anisotropy (see eq 1).

of very high affinity interactions and the corresponding volume change, ΔV, for the reaction. This technique has been used to characterize the free energy, ΔG, and volume changes, ΔV, involved in the dissociation of enolase (Paladini & Weber, 1981b), LDH (King & Weber, 1985), tryptophan synthetase (Silva et al., 1986), and the *lac* repressor (Royer et al., 1986).

When a solution of H2A–H2B dimers and $(H3)_2(H4)_2$ tetramers is mixed at an ionic strength of 2 M and at appropriate protein concentrations, two of the dimers bind to one tetramer to form the histone core octamer (Yager & van Holde, 1984). In the preceding paper (Royer et al., 1989), we followed this association as a function of salt concentration. We found that under certain conditions, isolated tetramers tended to form higher order aggregates. The aggregation properties depended on the type of protein. The isolated H3/H4 solution from the commercial calf thymus preparation was found to be octamer under all conditions tested, whereas the freshly prepared chicken erythrocyte H3/H4 dimerized to octamer either above 5 μM protein or in 2 M NaCl. At 2 M NaCl, H2A–H2B dimers from both preparations were able to displace one $(H3)_2(H4)_2$ tetramer from the octamer to form core particles. Since the preceding results may implicate mechanisms for nucleosome assembly and unfolding, we have extended the studies using high-pressure techniques in order to better quantitate the various histone subunit interactions and how these may be effected by ionic strength.

Materials and Methods

The preparation, characterization, labeling procedures, and instrumentation are described in the preceding paper (Royer et al., 1989). Hydrostatic pressure was applied using a cell based on the design of Paladini and Weber (1981a). L-format polarization values were corrected for pressure-induced birefringence by using the method of Paladini and Weber (1981a) with modifications (Royer and Scarlata, unpublished results). All measurements were done at 20 °C. The average error of the polarization values did not exceed ±0.002 while the errors in phase angle and modulation ratio were below ±0.200° and ±0.004, respectively. Pressure data were taken at 200-bar intervals (1 bar = 1.05 atm), allowing the system to thermally equilibrate approximately 5 min before taking data. Reversibility was checked by returning the pressure to atmospheric over a period of 20 min. The fluorescence parameters of individually labeled histones were completely reversible (within error) except where noted.

Data Analysis. Average spherical rotational volumes, $\langle V\rangle_m$, were calculated as before [see Royer et al. (1989)] from the raw polarization values (p) and average lifetimes ($\langle\tau\rangle$) through the Perrin equation:

$$\left(\frac{1/p_0 - 1/3}{1/p - 1/3}\right) - 1 = RT\langle\tau\rangle/\eta V_m \qquad (1)$$

where p_0 is the polarization is the absence of rotational motion, which for dansyl equals 0.4 (Weber, 1951), R is the gas constant, T is the absolute temperature, and η is the viscosity of the solvent. Water viscosity data under pressure were taken from Bridgeman (1970). The dissociation constants and volume changes from the pressure data were calculated as described by Paladini and Weber (1981a) and summarized as follows. The degree of oligomer dissociation at any pressure, α_p, can be calculated by using

$$\alpha_p = [1 + Q(A_p - A_m)/(A_o - A_p)]^{-1} \qquad (2)$$

where Q is the ratio of the quantum yield of the monomer to the oligomer, A_p is the anisotropy at pressure p, A_m is the anisotropy of the monomer, and A_o is the anisotropy of the oligomer. The equilibrium constant, K_d, for a monomer–dimer equilibrium can be expressed as

$$K_d = 4D_0[\alpha^2/(1 - \alpha)] \qquad (3)$$

and for a tetramer–monomer equilibrium:

$$K_d = 256T_0^3[\alpha^4/(1 - \alpha)] \qquad (4)$$

where D_0 and T_0 are the total protein concentration of dimer and tetramer, respectively. Since the derivative of the free energy change with respect to pressure corresponds to the volume change at constant temperature, we can relate the change in K_d from atmospheric pressure, K_{d_0}, to the K_d at pressure p, K_{d_p}, by

$$\ln K_{d_p} - \ln K_{d_0} = p\Delta V/RT \qquad (5)$$

Using the above expressions to plot either $\ln [\alpha^2/(1 - \alpha)]$ or $\ln [\alpha^4/(1 - \alpha)]$ as a function of pressure yields the atmospheric K_d from the y intercept and the volume change from the slope of the least-squares line between $\alpha = 0.1$ and 0.9, where no curvature of the plot exists.

The volume changes reported here have a maximum error of ±7 mL/mol and a 20% error for the K_d values calculated from the error in anisotropy values and the linear least-squares regression of these data.

Results

Pressure Dissociation of Chicken Erythrocyte (CE) H2A–H2B–DNS and Reconstituted Complexes. The application of high pressure leads to the dissociation of the CE dimers. The polarization of a 2 μM solution of dansylated CE dimer in 200 mM NaCl decreased 2-fold under pressure (Figure 1, left). Raising the protein concentration to 8 μM produces some aggregation, as seen by the higher polarization, and shifts the dissociation curve to higher pressures, indicating indeed that the transition corresponds to subunit dissociation. We note that at 2 kbar, the 2 μM sample is completely dissociated whereas, at the higher concentration, some dimer remains. The fluorescence lifetime was found to decrease slightly with pressure (11.5 to 10.8 ns in 2.0 kbar). The average rotational volume of the 2 μM solution decreased approximately 2-fold in the pressure range studied here, which is consistent with a dimer–monomer equilibrium.

Table I lists the changes in rotational volume, K_d, $C_{1/2}$, or concentration needed for 50% dissociation, and ΔV for the CE dimer experiments. From these data, we find that there may be a slight decrease in dimer stability in high salt. The rotational volumes of the CE protein listed in Table I at the two salt concentrations show that the local dansyl mobility in the

Table II: Dissociation Parameters for Dansyl-Labeled Histone H3/H4 Subunits

source	$[H3/H4]_T$ (μM)	[NaCl] (M)	K_d	equilibrium	$C_{1/2}$ (M)	ΔV_a[a] (mL/mol)	$\langle V\rangle_m$[a](atm) (mL/mol)	$\langle V\rangle_m$[a](2 kbar) (mL/mol)
CE	3.6	0.2	1.5×10^{-8} M	tetramer–dimer	1.5×10^{-8}	108 ± 4	46 200	27 600
CE	3.6	2.0	1.4×10^{-9} M	octamer–tetramer	1.4×10^{-9}	168 ± 6	58 300	37 800
CT	0.5	0.2	9×10^{-21} M^3	octamer–dimer	8×10^{-8}	143 ± 2	60 000	15 300
CT	0.5	2.0	1×10^{-21} M^3	octamer–dimer	4×10^{-8}	142 ± 2	46 000	13 000

[a] ΔV refers to the thermodynamic volume charge of the reaction, and $\langle V\rangle_m$ refers to the hydrodynamic volume extracted from the fluorescence anisotropy (see eq 1).

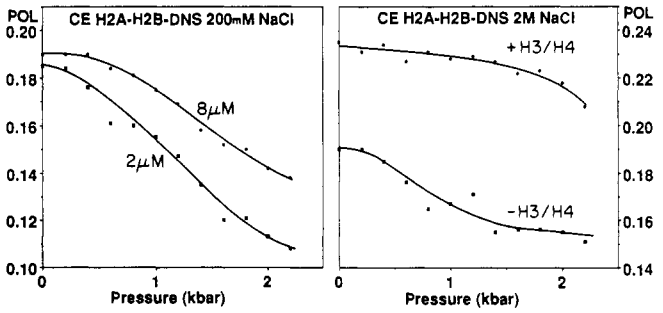

FIGURE 1: Polarization of the dansyl fluorescence vs pressure for (left) 2 and 8 μM chicken erythrocyte H2A–H2B–DNS preparations in 200 mM NaCl and (right) 2 μM H2A–H2B–DNS in the absence and presence of 43 μM unlabeled H3/H4 and 45 μM unlabeled H2A–H2B.

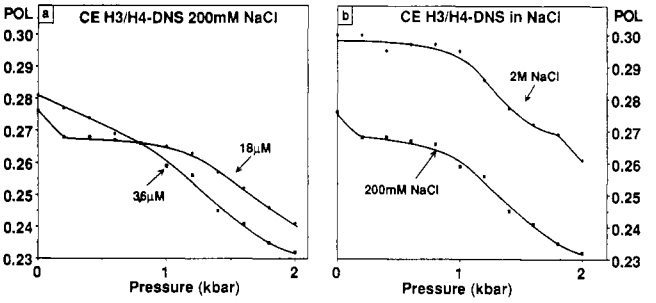

FIGURE 2: Polarization of the dansyl fluorescence vs pressure for (a) 3.6 and 18 μM chicken erythrocyte H3/H4–DNS in 200 mM NaCl and (b) 2.6 μM H3/H4–DNS in 200 mM and 2 M NaCl.

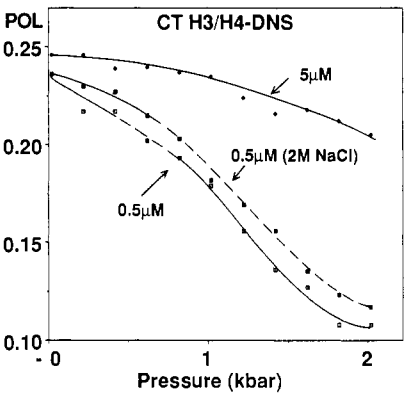

FIGURE 3: Polarization of the dansyl fluorescence vs pressure for 0.5 μM calf thumus H3/H4–DNS in 100 mM and 2 M NaCl and 5 μM H3/H4–DNS in 200 mM NaCl.

monomers is highly dependent on ionic strength.

Next, unlabeled H3/H4 and unlabeled CE core protein were added to CE H2A–H2B–DNS at 2 M NaCl to yield a total of 43 μM core histone. The polarization of the complex slowly decreases with pressure until 1.6 kbar where the slope changes to a larger negative value (Figure 1b). We interpret the low pressure-behavior as being due to a very small perturbing effect of pressure on the core particle due to a small volume change of association. Thus, the slope is shallow up to approximately 1.6 kbar. However, as dimer is dissociated from the core particle, it, too, is destabilized and with a much larger negative volume change. The slope becomes steeper with pressurization, resulting in more free dimer which can be dissociated. If we use the average dissociation constant of the two H2A–H2B dimers from the tetramer given by Benedict et al. (1984) and supported in the preceding paper (Royer et al., 1989), we find, using eq 5, that the volume change for dimer association with tetramer must indeed be very low (~18 mL/mol).

Pressure studies of CT H2A–H2B–DNS were also made. The pressure dissociation was concentration dependent and, in the presence of H3/H4, showed a behavior similar to its CE counterpart. However, because these samples showed only 80% pressure reversibility, dissociation constants are not reported.

Pressure Dissociation of the CT and CE H3/H4–DNS Complexes. Pressure studies of the CE H3/H4–DNS tetramer were first carried out at 200 mM salt (Figure 2a). At 3.6 μM H3/H4 tetramer, we find an initial drop in polarization within the first 200 bar, a plateau out to approximately 800 bar, and a second sigmoidal decrease. Upon a 5-fold increase in tetramer concentration, the curves for both transitions show a shift toward higher pressure, as would be predicted for a subunit dissociation process. In the previous paper (Royer et al., 1989), we found that the CE tetramer tended to form higher order aggregates above 5 μM at 200 mM salt, and we thus interpret the initial drop as corresponding to the dissociation of a small amount of aggregate to tetramer. The second transition gives rise to an approximate 2-fold decrease in the rotational volume (Table II), corresponding well to a tetramer to dimer transition. The K_d values calculated for H3/H4 pressure dissociation curves at two concentrations agree within the reported error. The K_d calculated from the data taken for the 3.6 μM solution was 1.5×10^{-8} M, whereas that calculated for the 18 μM solution was 1.2×10^{-8} M. All high-pressure parameters are listed in Table II.

Raising the salt concentration to 2 M has a dramatic effect on the pressure dissociation curve of CE H3/H4–DNS (Figure 2b). The initial rotational volume corresponds to a dimer of tetramers [see Royer et al. (1989)] which is stabilized by over 1.3 kbar, corresponding to approximately 6 kcal of stabilization per mole of octamer. Upon the application of hydrostatic pressure, the polarization decreases in a sigmoidal fashion to the value found for the tetramer, and then it begins to decrease again. We interpret the first transition as being that of the dissociation of the dimer of tetramers to tetramer since it reaches the plateau polarization value observed for the tetramer. The K_d calculated for the transition is 1.4×10^{-9} M, and the ΔV is 168 mL/mol. The tetramer dissociation to dimer is also stabilized by salt as evidenced by the shift of the beginning of this curve to 2.2 kbar in 2 M salt as opposed to 800 bar in 200 mM salt; although the transition is only beginning at 2.2 kbar, this shift in the onset of depolarization corresponds to a stabilization of at least 2 kcal/mol of tetramer.

Figure 3 shows the results obtained for the CT H3/H4–DNS. In the preceding paper (Royer et al., 1989), we found

that this protein formed aggregates of tetramers even at lower salt and protein concentrations. The large shift to higher pressure of the dissociation curve upon a 10-fold increase in concentration is indicative of the formation of even higher order aggregates. The value of the rotational volume for the 0.5 μM solution at atmospheric pressure corresponds to an octamer. Since the rotational volume decreases by a factor of 4, the pressure dissociation curve is interpreted as corresponding to an octamer to dimer transition. The K_d and ΔV were calculated assuming equivalence of an octamer–dimer transition to a tetramer–monomer transition. We note that, unlike the CE system, the octamer to tetramer and tetramer to dimer equilbria are not distinct (i.e., no plateaus) and thus cannot be treated separately. Raising the salt concentration from 200 mM to 2.0 M NaCl stabilized the complex by a factor of 10 in the K_d, but this effect is not nearly as large as that of the CE system. All pressure parameters are listed in Table II.

Discussion

In this study, we used high-pressure spectroscopy to examine histone subunit interactions. This technique allows for the equilibrium study of very high affinity protein subunit interactions. The concentration dependence of pressure transition confirms the destabilization of subunit interactions by pressure.

We first focus on the results obtained for the isolated CE H2A–H2B dimer. We have observed the concentration dependence of the CE dimer pressure profiles, confirming that, indeed, high pressure results in dimer dissociation. Using concentrations where only the dimer form was present, we have determined the dimer to monomer dissociation constant of 0.1 μM in 2 M NaCl. Using CD and intrinsic fluorescence, D'Anna and Isenberg (1974a) reported the dimer dissociation K_d to be approximately 1 μM at 2.0 M salt. We find only a very small destabilization by salt for the CE dimer, and thus do not believe that the difference in salt concentration can account for the 10-fold difference found for the dimer affinity. It is possible that, since the protein concentrations used by D'Anna and Isenberg were close to the concentrations where we observed some higher order aggregation, these investigators may have been following both the tetramer–dimer and the dimer–monomer equilibria. The lowering of the ionic strength to 200 mM may stabilize the CE dimer by a factor of 2 (approximately equivalent to the error in our measurements), yielding a K_d of 6×10^{-8} M. This value, however, is very close to the calculated for high salt, indicating a reasonable degree of reproducibility of the data. The rotational volume observed for the CE monomers in 200 mM NaCl (observed at at high pressures) is slightly lower than that predicted for the monomer molecular weight. At 2 M NaCl, however, the rotational volume is slightly larger than that value. Thus, while no large salt perturbations are observed for the dimer subunit interactions, addition of salt appears to have significant effect on the local environment around the dansyl residue in the monomer.

Monitoring the dansyl polarization, we found that high pressure had only a small destabilizing effect upon the reconstituted core particle as evidenced by the shift to much higher pressures of any decrease in polarization. The volume change for the dimer association to tetramer to form core octamer must therefore be much lower than those found for most proteins (Weber & Drickamer, 1983; Heremans, 1982). Solvent packing and electrostriction of exposed charges at the subunit interfaces are thought to be primarily responsible for the destabilization of subunit interactions under pressure. The X-ray structure of the core particle (Richmond et al., 1984; Burlingame et al., 1985) shows large solvent channels at the tetramer–dimer interface, as well as only three minor contacts between the H2B and the H4 subunits. The presence of these solvent channels serve to lessen the importance of solvent packing in the overall volume change for association, resulting in a much smaller value. Benedict et al. (1984) confirmed previous observations (Eickbush & Moudrianakis, 1978) that dimer association occurs with a small net proton release. This release was proposed to be caused by histidine–tyrosine or histidine–lysine interactions, and dimer–tetramer contacts are also stabilized by a number of weak hydrogen bonds (Eickbush & Moudrianakis, 1978). Hydrogen bond formation appears to occur with a very small decrease in volume, which would result in complex stabilization by pressure (Josefiak & Schneider, 1980), whereas production of a charge results in a large volume change due to electrostriction of water around charges (Hamann, 1980). Since the histidine side chain deprotonation does not result in a net change in charge ($NH^+ = N + H^+$), the small volume change is consistent with a balance between complex destabilization from water occupying free volumes and histidine–tyrosine interactions stabilized by hydrogen bonds.

Under pressure, we also observed the dissociation of the CE H3/H4 tetramer to dimers, which, on the basis of previous observations (Isenberg, 1979), we assume to be $(H3)_2$ and $(H4)_2$. The K_d values calculated for the two concentrations employed were well within a factor of 2, 1.5×10^{-8}, and 1.2×10^{-8} M. We have found that the $(H3)_2(H4)_2$ tetramer tends to aggregate to predominantly dimers of tetramers. The tendency of the H3/H4 tetramer to aggregate is different for the commercial calf thymus and freshly prepared chicken erythrocyte proteins. The calf thymus protein was in the octamer form under all conditions studied, whereas the chicken erythrocyte H3/H4 only formed octamer at either higher protein or salt concentrations. The difference may be attributed either to small variations in the sequences of the two proteins or to the method of preparation. The salt-induced aggregation and stabilization are interpreted to be caused by charge shielding. We found that the apparent dissociation constant of the CT octamer to dimer is similar to the tetramer to monomer value reported by D'Anna and Isenberg (1974b). The stabilization of the octamer by high salt in the calf thymus protein was 10-fold. We found the tetramer–dimer equilibrium for the CE H3/H4 to be approximately 15 nM. The octamer–tetramer transition was stabilized by approximately 6 kcal/mol by 2 M salt. The shift of the tetramer–dimer transition at high ionic strength was beyond the pressure range of our instrumentation. However, we estimate the lower limit of the stabilization to be approximately 2 kcal/mol of tetramer.

In this paper, and the preceding one (Royer et al., 1989), we have characterized and quantitated the observations of D'Anna and Isenberg (1974a,b) that histones have a strong tendency to self-associate and have found that this behavior is effected by the local ionic strength. It is interesting to interpret these results in light of nucleosome assembly where it has been observed that H3/H4 first binds to DNA and afterward H2A–H2B binds to the tetramer–DNA complex (Worcel et al., 1978; Jackson & Chalkley, 1981). If DNA acts upon the newly synthesized H3/H4 tetramer in a manner similar to high salt, then the presence of DNA could cause the tetramer to be more receptive to protein association. This may be helpful in positioning many tetramers along the DNA. Although the tetramer may self-associate, the tendency to form the core particle in the presence of H2A–H2B dimers is stronger than the self-association. The interactions between

the H3/H4 tetramers among themselves, as well as the interactions between the dimers themselves, may also be important to the regulation of nucleosome unfolding for transcription and replication. Since these interactions are highly sensitive to the local ionic strength, the magnitude of the changes in this latter need the DNA binding proteins which participate in replication and transcription. Using the results presented in this and the preceding paper (Royer et al., 1989) for the various histone subunit interactions, we are currently conducting similar studies to characterize histone–DNA interactions under a variety of conditions.

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