Thermodynamic Studies of the Core Histones: pH and Ionic Strength Effects on the Stability of the (H3−H4)/(H3−H4)$_2$ System†

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ABSTRACT: The self-associative behavior and the thermal stability of the H3/H4 histone complex was studied in low-ionic strength conditions by several physicochemical techniques, including differential scanning calorimetry and circular dichroism spectroscopy. At neutrality, the major molecular species present in solution is the (H3−H4)$_2$ tetramer. Its thermodynamic properties cannot be studied directly though, since its thermal denaturation is completely irreversible even at the lowest salt concentrations. However, a complete thermodynamic analysis can be performed at low ionic strength and pH 4.5, where the (H3−H4)$_2$ tetramer is quantitatively dissociated into two H3−H4 dimers and where almost complete reversibility of the thermal transitions is attained. The unfolding transition temperature of the 26.5 kDa H3−H4 dimer increases as a function of both the ionic strength of the solvent and the total protein concentration. The thermal denaturation of the H3−H4 dimer is characterized by the presence of a single calorimetric peak, centered at 58 °C, with a corresponding enthalpy change of 25 kcal/mol of a 13 kDa monomer unit and a change in heat capacity upon unfolding of about 0.6 kcal/(K mol of 13 kDa monomer unit). The complex between histones H3 and H4 (tetramer or dimer) is stable between pH 9.5 and 3.0. At pH 1.5, the system is almost completely unfolded at all temperatures. At low ionic strengths and pH values between 5.0 and 2.5, the H3−H4 dimer behaves as a highly cooperative system, melting as a single unit; i.e. individual H3 and H4 folded monomers are not detectable during the treatment. The two-state mechanism accounting for the unfolding of the H3−H4 dimer at pH 4.5 is the same as that described for the H2A−H2B dimer at neutrality. Just like for the H2A and H2B histones, the H3 and H4 polypeptides are properly folded only when assembled as H3−H4 dimers or in higher-order histone assemblies. Therefore, coupling along the interfaces of the two chains within the heterodimer is the major factor contributing to the stabilization of the secondary and tertiary structures of the chains as well as of the histone dimers.

Inside the nuclei of all eukaryotic cells, the DNA is complexed with histones and organized in the form of chromatin. It has been proposed (Kornberg, 1974) that two copies of each of the histones H2A, H2B, H3, and H4 are arranged in regular oligomeric assemblies, the core histone octamers, and together with approximately 200 bp[1] of DNA and one copy of H1 are organized in repeating structural units, the nucleosomes. The core histone octamer has been shown by a variety of physicochemical methods to be internally organized as a tripartite protein assembly (Eickbush & Moudrianakis, 1978; Arents et al., 1991). The nature of associations of the histone octamer with DNA in nucleosomes has been the main focus of several earlier studies on the thermal denaturation of the structural components of chromatin. Most of these studies employed either changes in DNA hyperchromicity, or circular dichroism and differential scanning calorimetry to monitor both DNA and protein components (Weischet et al., 1979; Bina et al., 1980; Olins et al., 1977; Simpson, 1979; Poon & Seligy, 1980; Dimitrov et al., 1988; Balbi et al., 1989; Carazza et al., 1991).

In an attempt to characterize the thermodynamic behavior of the components of the nucleosome in a more detailed and quantitative way, we have initiated a systematic study of the energetics of the core histone octamer alone or in association with DNA. We have presented (Karantza et al., 1995) the results of such a study on the H2A−H2B histone dimer, the simplest thermodynamic subunit of this system. The thermal stability of the H2A−H2B dimer was examined as a function of ionic strength and pH. In low-ionic strength conditions, and at pH values between 6.5 and 8.5, the H2A−H2B dimer undergoes a highly cooperative folding/unfolding transition without any detectable dissociated, yet folded, H2A and H2B monomers. The unfolding transition temperature increases as a function of ionic strength. The H2A−H2B dimer is a stable complex between pH 5.5 and 10.5. Its stability is greatly reduced outside this pH range, with complete unfolding taking place below pH 4.0.

In the present study, we examine systematically the thermodynamic behavior of the complex between the H3 and H4 core histones. Early studies on the association between histones H3 and H4 showed the formation of a very strong, 1:1 complex in a concentration dependent reversible association, which is almost exclusively shifted toward a four-chain

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[1] Abbreviations: ΔC_p, observed difference in the heat capacity between the unfolded and the native states; ΔH, enthalpy change; ΔH_{cal}, calorimetric enthalpy change; ΔS, entropy change; $[\Theta]_\lambda$, molar ellipticity in deg cm^2 dmol^{-1}; CD, circular dichroism; CM, (carboxymethyl)-cellulose; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; SSR, sum of the squared residuals of the fit; T_m, melting temperature; bp, base pairs.

assembly, i.e. the $(H3-H4)_2$ tetramer (D'Anna & Isenberg, 1974; Roark et al., 1974; Hyde & Walker, 1975; Rubin & Moudrianakis, 1975). A thorough analysis of the structural integrity of the H3−H4 complex at extreme conditions (4.0−8.0 M urea, low pH, i.e., 0.01 M HCl, etc.) has provided evidence for the separation of this complex even down to single polypeptide chains, with the predominant species being a dimer (Lewis, 1976). The author questioned whether the population of these dimers consisted of H3−H4 heterodimers or H3−H3 and H4−H4 homodimers or represented a mixture of all these. Such dismutations of the $(H3-H4)_2$ system were not seen at urea concentrations less than 2.0 M or at pH values greater than 4.0.

The fluctuations of the structure of the H3/H4 histone system in solution favoring a canonical tetramer as the starting state have been examined by several techniques, including NMR, CD, and ultracentrifugation (Moss et al., 1976; Bohm et al., 1977; Rubin & Moudrianakis, 1975; Godfrey et al., 1990; Baxevanis et al., 1991). The important role of the $(H3-H4)_2$ tetramer in core histone octamer and nucleosome assembly has been well established. Biochemical (Sollner-Webb & Felsenfeld, 1975; Eickbush & Moudrianakis, 1978; Ruiz-Carrillo & Jorcano, 1979; Daban & Cantor, 1982) and crystallographic data (Arents et al., 1991) support the model of a tripartite organization, in which a centrally located $(H3-H4)_2$ tetramer is flanked by two H2A−H2B dimers.

In order to examine the thermodynamic behavior of the H3/H4 system in solution, as a prerequisite step toward understanding the energetics of the nucleosome as a whole, we studied the thermal stability of the complex between histones H3 and H4 as a function of ionic strength and pH. The results of these experiments are presented in this paper and provide a thermodynamic explanation for previously reported biochemical and structural data concerning the stability of the complex between histones H3 and H4, and the first case of a stable (H3−H4) dimer in solution. The high degree of cooperativity seen upon thermal unfolding of the H3−H4 dimer mirrors the results seen with the H2A−H2B dimer as well as those of the tetrameric oligomerization domain of the p53 suppressor protein (Johnson et al., 1995). Both these proteins also undergo a two-state thermal unfolding transition between native oligomer and unfolded monomers. Such behavior underscores the importance of the contribution of the quaternary structure to the stabilization of the functional oligomeric unit.

MATERIALS AND METHODS

Isolation of Histones. Chicken erythrocyte histones were isolated by a modification of the salt extraction procedure previously used in this laboratory (Eickbush & Moudrianakis, 1978). The $(H3-H4)_2$ tetramer was efficiently separated from the H2A−H2B dimer on a CM-cellulose (Whatman CM-52) column equilibrated with 0.1 M potassium phosphate, 1 mM EDTA, and 0.5 M urea (pH 6.7) and eluted using a 0.1 to 0.6 M KCl step gradient. All isolation steps were performed at 4 °C, with repeated additions of PMSF.

Sample Preparation. Histone $(H3-H4)_2$ samples were dialyzed into the desired pH and NaCl- and AcONa-containing buffers overnight to assure dialysis equilibrium. The DSC experiments at pH 7.5 were performed in solutions containing 10−400 mM NaCl, 10 mM HEPES, or 10 mM imidazole. Samples for the DSC experiments at pH 4.5 were prepared in solutions containing 10 mM NaCl and 10 mM glycylglycine or 0−40 mM AcONa and 10 mM glycylglycine. Samples for the CD/ionic strength experiments were prepared in solutions containing 50−400 mM NaCl and 10 mM HEPES (pH 7.5) or 15−110 mM AcONa and 10 mM MES (pH 4.5). The temperature dependence of the CD spectra was monitored in solutions of 50 mM NaCl and 10 mM HEPES (pH 7.5) or 50 mM NaCl and 10 mM MES (pH 4.5). The CD/pH series experiments were performed in solutions containing 50 mM NaCl and ranging in pH from 1.5 to 9.5. The buffers used were 10 mM glycine for pH 1.5−4.0, 10 mM MES for pH 4.5−5.5, 10 mM HEPES for pH 6.5−7.5, and 10 mM bicine for pH 8.5−9.5. Samples for the CD measurements of the protein concentration series were prepared in 50 mM NaCl and 10 mM MES (pH 4.5). For the trypsin digestion experiments, $(H3-H4)_2$ samples were dialyzed against 100 mM NaCl, 1 mM EDTA and 10 mM HEPES (pH 7.5). For the small-zone exclusion chromatography experiments, H2A−H2B and $(H3-H4)_2$ samples were prepared in 50 mM NaCl, 1 mM EDTA, and 10 mM HEPES (pH 7.5), or 50 mM NaCl, 1 mM EDTA, and 10 mM glycylglycine (pH 4.5). The equilibrium sedimentation experiments were performed in 50 mM NaCl and 10 mM HEPES (pH 7.0) or 50 mM NaCl and 10 mM MES (pH 4.5). Protein concentrations used were 2 mg/mL for the DSC experiments, 0.3 mg/mL for the CD ionic strength and pH experiments, 0.1−1.6 mg/mL for the CD protein concentration series, 1 mg/mL for the trypsin digestion and the small-zone exclusion chromatography experiments, and 0.1−1.7 mg/mL for the equilibrium sedimentation experiments. The concentrations were determined spectrophotometrically immediately prior to performing experiments as previously described (Godfrey et al., 1990). Protein purity was assayed on 20% acrylamide−0.1% $NaDodSO_4$−polyacrylamide gels (Laemmli, 1970). Proteins were visualized by bathing the gels in 40% ethanol, 5% acetic acid, and 0.1% Coomassie Brilliant Blue R. Gels were subsequently destained with 20% ethanol and 5% acetic acid. Gels of protein preparations used in this study revealed only the appropriate core histones with no detectable breakdown products.

Differential Scanning Calorimetry. Experiments were performed at a scanning rate of 60°/h in the newly developed high-precision differential scanning microcalorimeter DS-92 (Biocalorimetry Center, The Johns Hopkins University, Baltimore, MD). The calorimetric unit was interfaced to an IBM PC microcomputer using an analog−digital converter (Data Translation DT-2801) for automatic data collection and analysis. Samples and reference solutions were properly degassed and carefully loaded into the calorimeter to eliminate bubbling effects. To test for the ability of the protein to renature, all samples were cooled at the end of the first scan, allowed to reequilibrate to the starting temperature, and then scanned again. The percent renaturation is expressed as the ΔH_{cal} of the second scan divided by that of the first. The excess heat capacity function was analyzed after normalization and base line subtraction using programs developed at the Biocalorimetry Center.

Circular Dichroism. All CD measurements were performed in a Jasco J-710 spectropolarimeter interfaced to an IBM PC microcomputer for automatic data collection and analysis. Temperature scans were performed by scanning

continuously from 6−80 °C in a 1 mm rectangular quartz cell (Hellma Scientific).

The temperature was controlled using a Haake PG20 temperature programmer interfaced to a Haake F3 circulating water bath, with a rate of increase in temperature of 60 °C/h. Temperature was monitored using a Microtherm 1006 thermometer and an S/N 117C temperature probe just outside of the sample cell. Data were collected using the time scan mode within the J-710 software package. The ellipticity at 222 nm was recorded every 20 s with a response time of 1 s and a band width of 1 nm. The ellipticity and the temperature were manually recorded at discrete intervals of 5 °C, and intermediate temperatures were interpolated for every intervening ellipticity reading to yield a complete description of Θ_{222} vs temperature. Ellipticity readings were normalized to fraction unfolded using the equation

$$P_u = (\Theta - \Theta_N)/(\Theta_D - \Theta_N)$$

where Θ_D and Θ_N represent the ellipticity values for the fully unfolded and fully folded state at each temperature, respectively, as calculated from the slopes of the base lines preceding and following the transition region.

Trypsin Digestion. $(H3-H4)_2$ tetramer was digested with trypsin at 5 μg/mL. The reaction proceeded overnight at 4 °C. At given times, aliquots were removed, treated with 2 mM PMSF, and processed for $NaDodSO_4$−polyacrylamide gel electrophoresis. The same procedure was performed for an $(H3-H4)_2$ sample, which had been heated up to 90 °C, at a heating rate of 60 °C/h, and cooled to 4 °C, prior to trypsin addition.

Small-Zone Exclusion Chromatography. The comparison of the self-associative properties of the H3−H4 complex in low-ionic strength conditions at pH 7.5 vs pH 4.5 was performed by the small-zone exclusion chromatography technique (Ackers, 1970), using Sephadex G-100 columns (1.7 × 100 cm) maintained at flow rates of 8−10 mL/h. The experiments were performed at room temperature, and 2 mL aliquots of the sample solutions were allowed to equilibrate at this temperature for 2 h before the columns were loaded. For the column elution profiles, 3 mL fractions were collected.

Equilibrium Sedimentation. Meniscus depletion sedimentation equilibrium runs were performed (courtesy of Dr. Michael Young) in a Beckman Model E analytical ultracentrifuge equipped with Rayleigh interference optics. Double-sector cells and a UV scanner were used. Sedimentation was performed at 21000−23000 rpm for 24 h at 23.8−24.9 °C. A partial specific volume, $\bar{\nu}$, of 0.720 mL/g was assumed for the H3−H4 histone complex.

RESULTS

Solutions of the histone $(H3-H4)_2$ tetramer tend to aggregate as the ionic strength and/or the protein concentration are raised. Even in low-ionic strength sulfate or phosphate solutions, the $(H3-H4)_2$ tetramer can form oligomers, which are smaller in size than those formed in the presence of sodium chloride. Aggregation of the $(H3-H4)_2$ tetramer is prevented by the addition of the histone H2A−H2B dimer, which acts as a "molecular cap" and regulates the assembly pathway toward the formation of tripartite octamers (Baxevanis et al., 1991). The self-associative behavior of the $(H3-H4)_2$, in the absence of the H2A−H2B dimer, makes it very difficult to analyze its thermodynamic properties under conditions where its interaction with the H2A−H2B dimer is particularly sensitive (Eickbush & Moudrianakis, 1978).

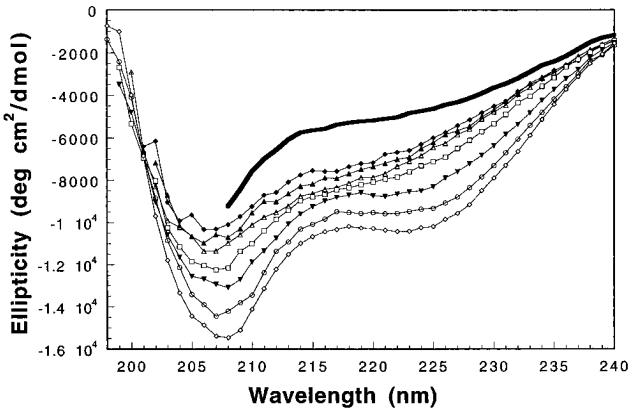

FIGURE 1: Far-UV CD spectra of the $(H3-H4)_2$ tetramer as a function of temperature. The temperatures are, from bottom to top, 20 °C (open diamonds), 30 °C (open circles), 40 °C (filled inverted triangles), 50 °C (open squares), 60 °C (open triangles), 70 °C (filled triangles), and 80 °C (filled diamonds). The experiments were performed at pH 7.5 in 50 mM NaCl and 10 mM HEPES. The thick solid line represents the 80 °C spectrum at pH 1.5 in 50 mM HCl and 10 mM glycine.

We decided to study the H3/H4 assembly system by differential scanning calorimetry (DSC) and circular dichroism (CD) spectroscopy, starting with a family of high-sensitivity calorimetric scans at pH 7.5 and low-ionic strength solutions (10−400 mM NaCl). Under these conditions and at room temperature, the predominant molecular species present in solution is the $(H3-H4)_2$ tetramer (Baxevanis et al., 1991). At this pH, all DSC scans were irreversible, even in the lowest salt concentration tried. The denaturation profiles were characterized by a large leading edge and a sharp trailing edge that in many cases made the definition of the base line after the thermal transition very difficult and the results irreproducible (data not shown), all indicative of a complex behavior. In addition, the apparent transition temperature decreased with protein concentration, despite the fact that the $(H3-H4)_2$ tetramer is a system in associative equilibrium of $2(H3-H4) \leftrightarrow (H3-H4)_2$, for which an increase in protein concentration would have been predicted to drive tetramer formation and, consequently, elevation of the transition temperature.

A series of CD experiments were also performed under similar conditions of low ionic strength and neutral pH. Wavelength scans at increasing temperatures showed the persistence of secondary structure even at high temperatures (Figure 1). The secondary structure exhibited a slow, progressive decrease between 20 and 50 °C, but it remained almost unchanged between 50 and 60 °C. Further heating to 90 °C did not contribute to any significant additional change in secondary structure. Upon the structure cooling, complete irreversibility was observed, with the protein remaining "locked" in the conformation it attained during the heating process. In solutions of low salt concentrations, the high-temperature conformational state of the $(H3-H4)_2$ tetramer in neutral pH was actually quite different from the high-temperature state of the H3−H4 complex in pH 1.5, as shown in Figure 1. As it is well established (Isenberg, 1979), histones are completely denatured in dilute acids, and therefore, the CD scan of the pH 1.5 solution can be assumed

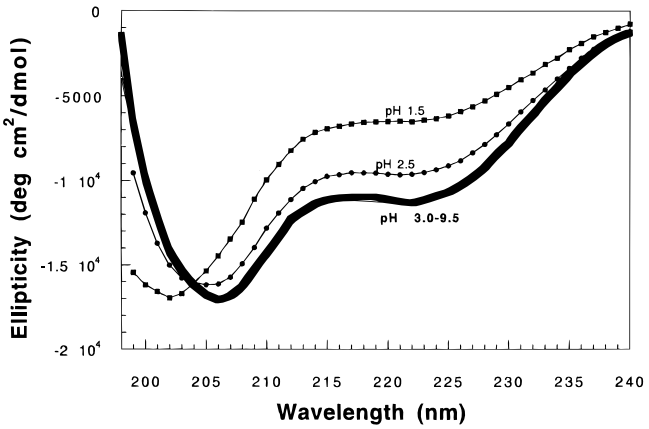

FIGURE 2: Far-UV CD spectra of the complex between histones H3 and H4 at different pH values. Experiments were performed in 50 mM NaCl and a 10 mM appropriate pH buffer. The protein concentration was kept constant at 22.6 μM 13 kDa monomer unit. The pH values are 3.0−9.5 (thick solid line), 2.5 (circles), and 1.5 (squares).

to represent the completely unfolded state of the complex between histones H3 and H4.

From the DSC and the CD data, it became obvious that, upon being heated at neutral pH and low ionic strength, the $(H3-H4)_2$ tetramer does not denature to a completely unfolded state. Instead, after an initial loss of some secondary structure, a partially unfolded state is attained and this state cannot be melted by a further increase in temperature. In addition, this state exhibits a tendency to aggregate as indicated by the observed decrease in the apparent denaturation temperature with increasing protein concentration. In some cases where the temperature scans were continued up to about 100 °C, protein precipitation was observed.

The possibility that the $(H3-H4)_2$ tetramer might aggregate during thermal unfolding at neutrality was further tested by limited trypsin digestion and comparison of the sensitivities to trypsin cleavage of the native $(H3-H4)_2$ and the putative aggregated state formed upon heating. In contrast to the native state of the $(H3-H4)_2$ tetramer (in low ionic strength, neutral pH, and room temperature) which is particularly sensitive to trypsin digestion, the high-temperature protein structure exhibits increased protection to cleavage (several trypsin-stable bands were observed), consistent with a state of complexity or aggregation higher than that present at the lower temperature (data not shown).

Since the histone tetramer exhibited nonideal behavior at neutrality, a series of calorimetric scans were performed in low ionic strength at pH 4.5, where better than 90% reversibility was observed and a meaningful thermodynamic analysis could be applied. We also decided to investigate by CD spectroscopy the secondary structure of the protein at pH 4.5 and compare it to that at pH 7.5. A series of wavelength scans at pH values ranging from 9.5 (solubility limit) to 1.5 in low-ionic strength solutions revealed that the secondary structure remains essentially the same between pH 9.5 and 3.0, whereas it decreases substantially at pH 2.5 and is almost completely lost at pH 1.5 (Figure 2).

The comparison of the quaternary structures of the H3/H4 system at pH 4.5 and 7.5 was performed by small-zone exclusion chromatography (Ackers, 1970) and equilibrium sedimentation. The G-100 elution profiles under low-ionic strength conditions, and pH 7.5 or 4.5, are represented by

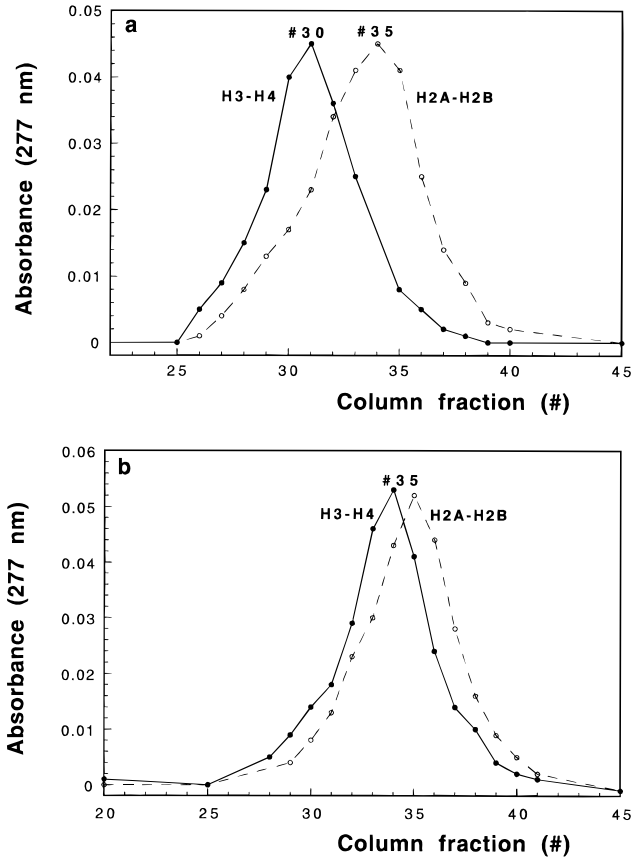

FIGURE 3: Small-zone gel filtration experiments (a) in 50 mM NaCl, 1 mM EDTA, and 10 mM HEPES (pH 7.5) and (b) in 50 mM NaCl, 1 mM EDTA, and 10 mM glycylglycine (pH 4.5). The elution profiles of the $(H3-H4)_n$ complex (n is either 1 or 2) and the H2A−H2B dimer are represented by the solid line and the dotted line, respectively, for both figures.

the solid lines in panels a and b of Figure 3, respectively. Analysis of the protein content under the peak areas by SDS−PAGE electrophoresis revealed equimolar amounts of histones H3 and H4. As a control, histone H2A−H2B dimer was fractionated on the same G-100 columns under the same conditions, and its elution profiles are represented by the dotted lines in panels a and b of Figure 3. At pH 7.5 (Figure 3a), as expected, the H2A−H2B dimer (28 kDa) elutes later than the $(H3-H4)_2$ tetramer (53 kDa). However, at pH 4.5 (Figure 3b), the two elution profiles almost coincide. These results considered together suggested that at pH 4.5 and low ionic strength the actual molecular species present in solution is the H3−H4 dimer, and not the $(H3-H4)_2$ tetramer. Therefore, at pH 4.5, the equilibrium $2(H3-H4) \leftrightarrow (H3-H4)_2$ is quantitatively shifted to the left, unlike what is known about the association complex between histones H3 and H4 under most conditions studied previously. The dissociation of the $(H3-H4)_2$ tetramer into two H3−H4 dimers does not involve any significant secondary structure reorganization of the separating subunits, since the CD spectra at pH 7.5 and 4.5 are practically superimposable, as mentioned earlier. This finding precludes any dismutation of the $(H3-H4)_2$ to the other possible dimers, i.e., H3−H3 and H4−H4, since this requires first the generation of free, monomeric H3 and H4 chains as intermediates. Such intermediates would be unstructured (Karantza et al., 1995; this study), and their appearance would be detectable by a loss in secondary structure, something that requires a pH lower than 3.0 under our experimental conditions. This interpretation is consistent

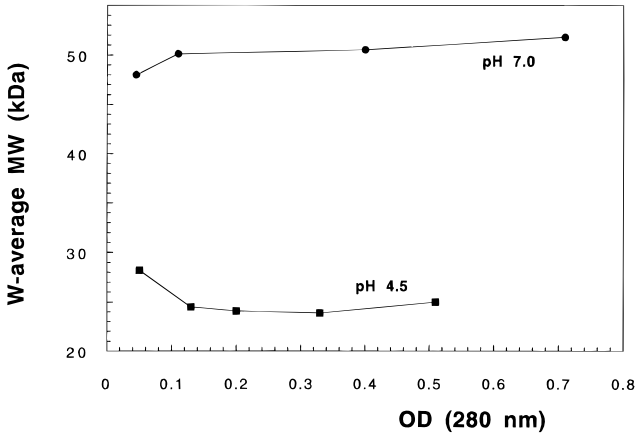

FIGURE 4: Meniscus depletion sedimentation of the complex between histones H3 and H4 at different pH values. The experiments were performed in 50 mM NaCl and a 10 mM appropriate pH buffer. The pH values are 7.0 (circles) and 4.5 (squares).

with earlier findings from the thorough experiments of Lewis (1976). Furthermore, we tested for the presence of such homodimers by native acrylamide electrophoresis in the GABA/acetate system with a running pH of 4.5. The only molecular species present was the H3−H4 dimer in equilibrium with small amounts of $(H3-H4)_2$ tetramer (data not shown).

The dissociation of the $(H3-H4)_2$ tetramer into two H3−H4 dimers at pH 4.5 and low ionic strength was further confirmed by equilibrium sedimentation experiments. Histone $(H3-H4)_2$ tetramer was dialyzed into low-ionic strength solutions at pH 4.5 and 7.0 and subjected to high-speed (meniscus depletion) equilibrium sedimentation in order to determine the number-average and weight-average molecular mass moments as a function of protein concentration within the ultracentrifuge cell. As shown in Figure 4, at pH 7.0, the weight-average molecular mass is essentially constant at about 51 kDa, indicating that at neutrality the predominant molecular species present in a low-ionic strength solution is the $(H3-H4)_2$ tetramer. However, at pH 4.5, the weight-average molecular mass is approximately halved, being essentially constant at about 24 kDa, which indicates that now the main molecular species present is indeed the H3−H4 dimer.

Ionic Strength Dependence of the Melting of the H3−H4 Dimer. Since the histone H3/H4 assembly system exhibits reversible unfolding behavior only when separated into two H3−H4 dimers, a thermodynamic analysis of this H3−H4 dimer was ultimately performed by differential scanning calorimetry and circular dichroism spectroscopy. The ionic strength dependence of the melting of the H3−H4 dimer at pH 4.5 was examined by DSC in solutions ranging from 0 to 40 mM sodium acetate (Figure 5a,b). As shown in Figure 5a, at pH 4.5 and very low-ionic strength conditions (15 mM AcONa), the unfolding transition of the H3−H4 dimer is characterized by the presence of a single calorimetric peak located at about 62 °C, an enthalpy change of about 27 kcal/mol of 13 kDa monomer unit, and a heat capacity difference between the unfolded and the native state (ΔC_p) of about 0.5 kcal/(K mol of 13 kDa monomer unit). The transition temperature increases from 58.2 °C at 10 mM acetate to 66.9 °C at 40 mM acetate (Figure 5b). The transition was better than 90% reversible in all cases examined. The enthalpy change shows an increase parallel with ionic strength (Figure

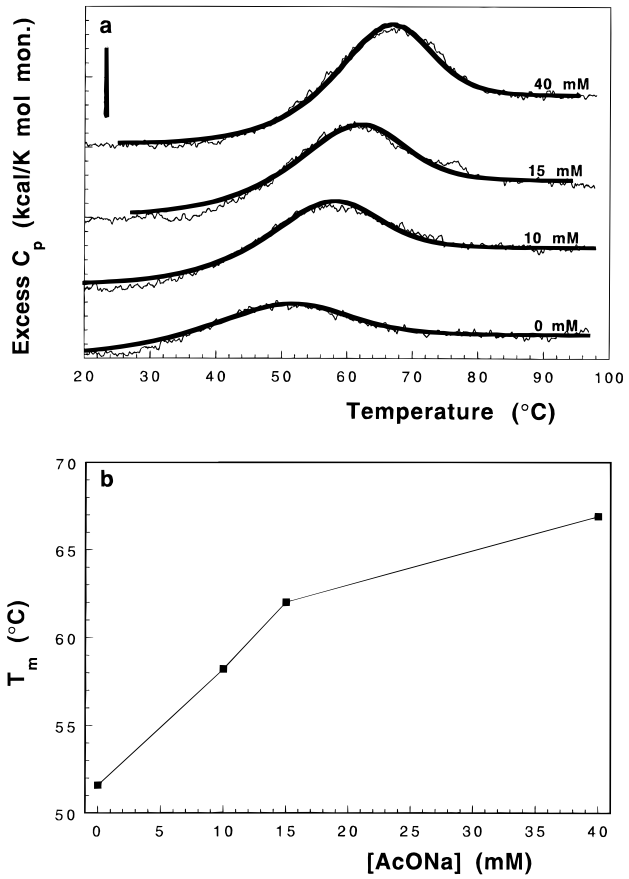

FIGURE 5: (a) Excess heat capacity of the H3−H4 dimer as a function of ionic strength. The AcONa concentrations are, from bottom to top, 0, 10, 15, and 40 mM. Experiments were performed in 10 mM glycylglycine (pH 4.5). The protein concentration was kept constant at 151 μM 13 kDa monomer unit. Experimental data are represented by thin solid lines. Theoretical curves generated with the parameters shown in Table 1 are represented by thick solid lines. For clarity of presentation, the data sets have been offset by 1 kcal/(K mol) below the 40 mM AcONa data set. The vertical bar equals 1 kcal/(K mol). (b) Melting temperatures of the H3−H4 dimer as a function of ionic strength using differential scanning calorimetry. Experiments were performed in 0−40 mM AcONa and 10 mM glycylglycine (pH 4.5). T_m represents the temperature corresponding to $C_{p,max}$.

5a). The straight line relationship between ΔH_{cal} and T_m ($r = 0.99$) yields a ΔC_p of 0.7 kcal/(K mol of 13 kDa monomer unit), or 0.05 cal/(g K), which is close to the one measured directly from individual scans and in the same order of magnitude, although somewhat smaller, as those previously reported for other proteins (Privalov & Makhatadze, 1990; Gomez et al., 1995). The slightly lower ΔC_p value for the H3−H4 dimer is discussed later in this paper. The unfolding transition is coupled to the dissociation of the two subunits of the H3−H4 dimer, as indicated by the concentration dependence of the transition temperature, described in a following paragraph. This effect is also reflected by the asymmetry (slight) of the heat capacity function which in all cases is skewed toward the low-temperature side of the transition, as expected for a transition coupled to dissociation (Freire, 1989).

A similar family of CD temperature scans at constant wavelength was performed on samples dialyzed against 15 to 110 mM sodium acetate (data not shown). The stability of the H3−H4 dimer increases as a function of ionic strength (Table 1). The transition temperature increases from 42.0

Table 1: Thermodynamic Parameters of H3−H4 Unfolding as a Function of Ionic Strength

		[salt] (mM)	T_m (°C)	$T°$ (°C)[a]	ΔH ($T°$) [kcal (mol monomer)$^{-1}$]	ΔS ($T°$) [cal (K mol monomer)$^{-1}$]	ΔC_p ($T°$) [kcal (K mol monomer)$^{-1}$]	SSR[b]
DSC	NaCl	10	58.0	84.7	40.8	114	0.6	45
	AcONa	0	51.6	86.9	30.9	85.7	0.3	44
		10	58.2	87.3	38.4	106	0.5	59
		15	62.0	89.5	40.8	112	0.5	72
		40	66.9	90.5	47.5	130	0.7	50
CD	AcONa	15	42.0	79.7	32.5	92.1		0.02
		37	49.3	86.1	40.9	114		0.02
		73	54.1	89.9	41.6	114		0.02
		110	56.0	92.2	44.1	121		0.03

[a] $T°$ is the reference temperature at which the intrinsic free energy $\Delta G°$ is equal to zero. [b] SSR is the sum of the square of the residuals.

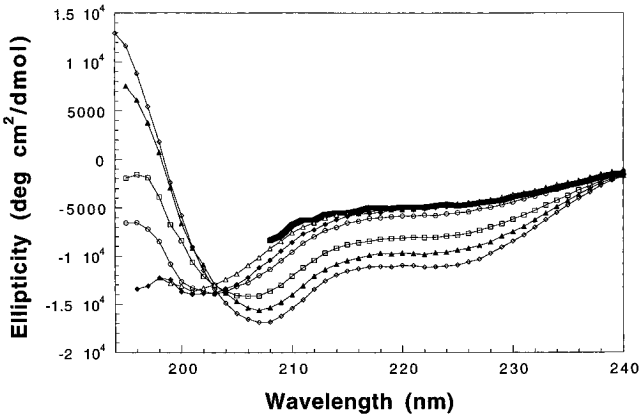

FIGURE 6: Far-UV CD spectra of the H3−H4 dimer as a function of temperature. The temperatures are, from bottom to top, 20, 40, 50, 60, 70, and 80 °C. Experiments were performed at pH 4.5 in 50 mM NaCl and 10 mM MES. The thick solid line represents the 80 °C spectrum at pH 1.5 in 50 mM HCl and 10 mM glycine.

°C at 15 mM acetate to 56.0 °C at 110 mM acetate. Although the absolute values of the range of the transition temperature are lower here due to the lower protein concentrations used, the trend of the change is very similar to the one observed in the DSC experiments.

Circular dichroism was also used to study the nature of the thermal unfolding of the H3−H4 dimer at pH 4.5. For this purpose, the CD spectra in the 190−240 nm region were recorded as a function of temperature. Figure 6 shows the family of temperature curves obtained with the protein in 50 mM NaCl. The content of α-helix is maximal at temperatures below the transition, whereas it decreases to zero at temperatures above the transition region, indicating that the thermal unfolding of the H3−H4 dimer at pH 4.5 is complete, with no secondary structure left present in the final high-temperature state. It should be noticed that a single well-defined isodichroic point is observed at 204 nm, which is consistent with the behavior of a system with only two, optically distinguishable, conformations. Further confirmation of the fact that the H3−H4 dimer undergoes a complete thermal denaturation at low ionic strength and pH 4.5 comes from the observation that the high-temperature wavelength scan of the H3−H4 complex in pH 4.5 is almost superimposable onto the high-temperature wavelength scan of the H3−H4 dimer at pH 1.5, which is represented by the thick solid line in Figure 6. As already mentioned, all histones are completely unfolded in dilute acidic solutions.

pH Dependence of the Melting of the H3−H4 Dimer. The thermal unfolding behavior of the H3−H4 dimer at low ionic strength was examined by circular dichroism spectroscopy in solutions ranging in pH from 5.0 to 1.5, and the results

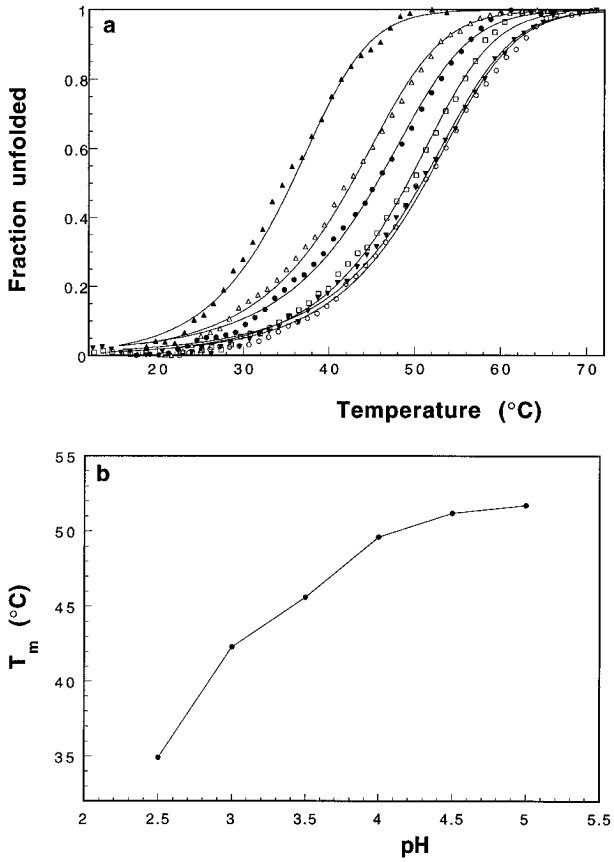

FIGURE 7: (a) Fraction of the H3−H4 dimer unfolded at different pH values as a function of temperature. The pH values are 5.0 (open circles), 4.5 (filled inverted triangles), 4.0 (open squares), 3.5 (filled circles), 3.0 (open triangles), and 2.5 (filled triangles). Experiments were performed in 50 mM NaCl and a 10 mM appropriate pH buffer. The protein concentration was kept constant at 22.6 μM 13 kDa monomer unit. The fraction unfolded was monitored by the ellipticity at 222 nm using CD spectroscopy. Experimental data are represented by symbols. Theoretical curves generated with the parameters shown in Table 2 are represented by solid lines. (b) Melting temperatures of the H3−H4 dimer as a function of pH using CD spectroscopy. T_m represents the temperature at which the fraction unfolded is equal to 0.5.

are shown in Figure 7a. The T_m of the H3−H4 dimer is essentially constant between pH 5.0 and 4.0 (at about 50 °C), whereas it progressively decreases below pH 3.5 (Figure 7b). The decrease in T_m, although initially gradual, becomes more pronounced at pH 2.5. The enthalpy change follows a similar pattern in that it remains essentially constant between pH 5.0 and 4.0 (at about 25 kcal/mol of 13 kDa monomer unit), whereas it decreases below pH 3.5 (Table 2). This is the expected behavior since enthalpy change is only temperature and not pH dependent.

Table 2: Thermodynamic Parameters of H3−H4 Unfolding as a Function of pH

pH	T_m (°C)	T° (°C)[a]	ΔH (T°) [kcal (mol monomer)$^{-1}$]	ΔS (T°) [cal (K mol monomer)$^{-1}$]	SSR[b]
2.5	34.9	70.0	36.4	106	0.04
3.0	42.3	79.2	39.0	111	0.02
3.5	45.6	81.4	41.4	117	0.02
4.0	49.6	83.8	42.2	118	0.02
4.5	51.2	86.4	43.4	121	0.02
5.0	51.7	85.5	43.4	121	0.02

[a] $T°$ is the reference at which the intrinsic free energy $\Delta G°$ is equal to zero. [b] SSR is the sum of the square of the residuals.

Circular dichroism experiments described earlier in this paper indicate that the α helical content of the complex between histones H3 and H4 (tetramer or dimer) as a function of pH follows a pattern that parallels the pH dependence of the T_m of the H3−H4 dimer unfolding transition. As already shown in Figure 2, the α helical content of the complex between histones H3 and H4 is maximum and essentially constant between pH 9.5 and 3.0, whereas it decreases at pH 2.5. An even more radical decrease in the α helical content of the H3−H4 dimer takes place below pH 2.0. A single well-defined isodichroic point is observed at 204 nm, just like during the thermal denaturation of the H3−H4 dimer described above (Figure 6).

Protein Concentration Dependence of H3−H4 Dimer Melting. The thermal unfolding behavior of the H3−H4 dimer as a function of protein concentration was examined by CD spectroscopy in solutions of low ionic strength at pH 4.5. The concentration of the protein was varied between 10 and 120 μM (as computed per 13 kDa monomer polypeptide subunit). The thermal denaturation profiles are shown in Figure 8a. It is clear that the T_m of the thermal unfolding (shown in Figure 8b) increases from 51.5 to 61.7 °C as the protein concentration is increased from 10 to 120 μM. This result is in agreement with a system that undergoes dissociation upon unfolding (Freire, 1989); in this case, an increase in protein concentration leads to higher levels of association and, therefore, to an increase in the stability and the melting temperature of the system.

Statistical Thermodynamic Analysis. The folding/unfolding transition of the H3−H4 dimer is characterized by the presence of a single peak and is reminiscent of the thermal behavior of the H2A−H2B dimer, which has been shown to be a highly cooperative system, melting as a single unit without any detectable intermediates of dissociated, yet folded, H2A and H2B monomers (Karantza et al., 1995). In order to investigate if the transition of the H3−H4 dimer also involves only two states, the calorimetric data were analyzed in a manner analogous to that described for the histone H2A−H2B dimer. The experimental excess heat capacity data (ΔC_p vs temperature) were analyzed in terms of the three-state formalism using a nonlinear least squares procedure as described elsewhere (Ramsay & Freire, 1990). In all cases in the low-ionic strength environment at pH 4.5, it was found that the population of intermediate conformations was insignificant and did not show any detectable contribution to the obtained calorimetric signal. Therefore, the thermal unfolding of the histone H3−H4 dimer could be precisely accounted for by a two-state mechanism, in which the only states highly populated, at all temperatures,

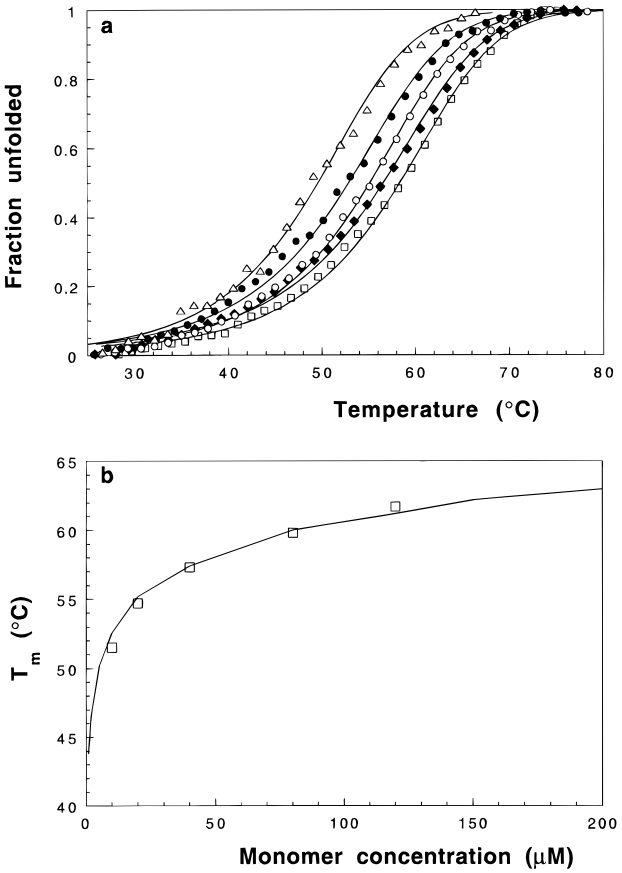

FIGURE 8: (a) Fraction of the H3−H4 dimer unfolded at increasing protein concentration as a function of temperature. The 13 kDa monomer unit concentrations are 10 μM (open triangles), 20 μM (filled circles), 40 μM (open circles), 80 μM (filled diamonds), and 120 μM (open squares). Experiments were performed in 50 mM NaCl and 10 mM MES (pH 4.5). The fraction unfolded was monitored by the ellipticity at 222 nm using CD spectroscopy. Experimental data are represented by symbols. Theoretical data generated with the parameters shown in Table 3 are represented with solid lines. (b) Melting temperatures of the H3−H4 dimer as a function of monomer unit protein concentration. The squares represent CD data. The solid line is a best-fit logarithmic curve through theoretical transition temperatures, as calculated according to the model that predicts the coupling between dissociation and unfolding of the H3−H4 dimer.

were native dimer and unfolded monomers. In such a case, the population of unfolded monomers, at any temperature, is described by the equation (Thomson et al., 1993)

$$P_U = P_{H3} + P_{H4} = K[(K^2 + 4)^{1/2} - K)]/2$$

where

$$K = \exp(-\Delta G°/RT)/(2[P_T])^{1/2}$$

The equilibrium constant, K, is a function of both the protein concentration, $[P_T]$, and the intrinsic free energy of stabilization, $\Delta G°$. All thermodynamic parameters in this paper are expressed on a per mole of individual polypeptide basis. The intrinsic Gibbs energy is described in a standard way as

$$\Delta G° = \Delta H°(T°) + \Delta C_p(T - T°) - T[\Delta S°(T°) + \Delta C_p \ln(T/T°)]$$

where, by definition, $T°$ is the temperature at which $\Delta G°$ is

Table 3: Thermodynamic Parameters of H3−H4 Unfolding as a Function of Protein Concentration

13 kDa monomer concn [mM]	T_m (°C)	$T°$ (°C)[a]	ΔH ($T°$) [kcal (mol monomer)$^{-1}$]	ΔS ($T°$) [cal (K mol monomer)$^{-1}$]	SSR[b]
10	51/5	85.0	41.7	116	0.04
20	53.2	86.4	53.4	121	0.02
40	58.3	87.0	46.5	129	0.02
80	59.8	87.4	44.5	123	0.02
120	61.7	88.1	43.6	121	0.03

[a] $T°$ is the reference temperature at which the intrinsic free energy $\Delta G°$ is equal to 0. [b] SSR is the sum of the square of the residuals.

equal to zero. It should be noted that this temperature is independent of protein concentration and does not correspond to the transition temperature, T_m, which is concentration dependent. Also, because of the asymmetry exhibited by a transition coupled to dissociation, the temperature of a maximum in the heat capacity function does not coincide with the temperature of half-completion of the denaturation transition. In this paper, the reported T_m values correspond to the temperature of the maximum in the heat capacity function. The thick solid lines in Figure 5a correspond to the theoretical curves, with the best-fit parameters to the experimental data analyzed by the nonlinear squares procedure mentioned above. The ionic strength series of CD experiments were also analyzed in terms of the above formalism.

The fitted thermodynamic parameters associated with the unfolding of the histone H3−H4 dimer, as a function of ionic strength at pH 4.5, have been summarized in Table 1 for both the DSC and CD experimental series. Similar parameter values were obtained from the analysis of the calorimetric and spectroscopic data. For example, at about 40 mM AcONa at $T°$, the enthalpy change measured calorimetrically is 47.5 kcal/mol of 13 kDa monomer unit compared to the value of 41.6 kcal/mol of 13 kDa monomer unit measured spectroscopically. The entropy change determined by DSC is 130 cal/K mol of 13 kDa monomer unit, and the one determined by CD is 114 cal/K mol of 13 kDa monomer unit. The corresponding $T°$ value determined by DSC is 90.5 °C, while that determined by CD is equal to 86.1 °C. The small difference of 4.4 °C is most likely due to the existence of temperature gradients and/or heat losses in the spectropolarimeter, since the control and measurement of temperature in this instrument are not as precise as in the calorimeter.

The two-state model was also used for fitting the spectroscopic data as a function of pH and protein concentration. The fitted thermodynamic parameters associated with the unfolding of the histone H3−H4 dimer between pH 5.0 and 1.5 are summarized in Table 2, whereas the thermodynamic parameters associated with the unfolding of the H3−H4 dimer as a function of protein concentration are presented in Table 3. As it was observed for the low-ionic strength series at pH 4.5, likewise, the pH series and the protein concentration series of the histone H3−H4 dimer show a two-state folding/unfolding transition; the only states populated at all temperatures are those of the native dimer and the unfolded monomers. The values of the parameters obtained from the spectroscopic measurements of the pH and protein concentration effects were comparable between them and to those obtained from the calorimetric and spectroscopic low-ionic strength data.

The model developed above and the resulting parameters accurately describe the protein concentration dependence of the transition temperature, as shown in Figure 8b in which the transition temperatures from the CD data series have been plotted as a function of monomer protein concentration. Therefore, the validity of the model that predicts the coupling between unfolding and dissociation of the chains of the H3−H4 dimer was checked both by the goodness of the fit of the DSC and CD data and by the protein concentration dependence of the T_m. As in the case of the H2A−H2B dimer, cooperative interactions within the H3−H4 dimer are the most important contributors to the stability of the system. Although the isolated H3 and H4 monomers are intrinsically unstable and cannot exhibit any autonomous degree of folding, their association is the driving force for the observed thermodynamic stability of the H3−H4 dimer. The high cooperativity of the system is consistent with the fact that the individual H3 and H4 monomers cannot be detected as independent entities in solution but have a strong tendency to form large aggregated structures, even at low-ionic strength conditions (Shih & Bonner, 1970; Olins & Olins, 1971; Ansevin & Brown, 1971; Shih & Fasman, 1971).

DISCUSSION

The fact that the H2A−H2B and H3−H4 dimers share a common mode of unfolding, i.e. a two-state, highly cooperative mechanism, is not very surprising if one takes into account the three-dimensional organization of these proteins. Although the individual histones do not exhibit significant primary sequence homology (they are only 4−6% identical), they all show a very similar secondary and tertiary spatial arrangement, known as the histone fold (Arents et al., 1991, 1995). For all four core histones, i.e. H2A, H2B, H3, and H4, the major portion of their ordered residues consists of a long central helix flanked on either end by a shorter helix and a loop and β-strand segment. Not only are the structures of the four individual core histones very similar to one another, but also the way these polypeptides associate to form the two biologically relevant types of dimers, i.e. the H3−H4 and H2A−H2B dimers, is highly conserved. Within both the H3−H4 and H2A−H2B dimer domains, the pairwise association of the folded histone chains follows a characteristic "handshake" motif, which results in an extensive molecular contact interface between the interacting polypeptides (Arents et al., 1991). As a consequence of the common dimerization motif, the path of the chains in the H3−H4 dimer is nearly identical to the analogous path in the H2A−H2B dimer. Therefore, the common folding/unfolding mechanism for the H3−H4 and H2A−H2B dimers is well substantiated by the strong similarities between the structural data sets for the two dimeric proteins.

As mentioned earlier, the heat capacity change upon unfolding of the H3−H4 dimer in pH 4.5 is about 0.7 kcal/K mol of 13 kDa monomer unit, or 0.05 cal/g K. For most up-to-date calorimetrically studied proteins, the ΔC_p ranges between approximately 0.09 cal/g K (for ribonuclease) to about 0.16 cal/g K (for myoglobin), the higher ΔC_p value being associated with a more hydrophobic protein core (Privalov & Makhataze, 1990; Gomez et al., 1995). Therefore, the heat capacity change exhibited during the thermal denaturation of the H3−H4 dimer, although on the same general order of magnitude, appears to be significantly lower than expected. A low ΔC_p value can be accounted for by

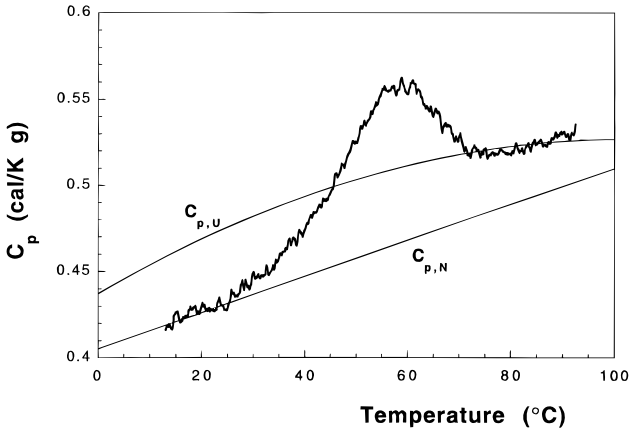

FIGURE 9: Partial specific heat capacity of the H3−H4 dimer as a function of temperature. The experiment was performed in 10 mM NaCl and 10 mM glycylglycine (pH 4.5) at a protein concentration of 150 μM 13 kDa monomer unit. The solid lines represent the heat capacities of the native ($C_{p,N}$) and unfolded ($C_{p,U}$) states, respectively. The heat capacity of the native state was calculated by linear extrapolation of the heat capacity of the low-temperature region. The heat capacity of the unfolded state was calculated using the method of Gomez et al. (1995) for a fully hydrated peptide of similar amino acid sequence. The experimental value for the unfolded state agrees well with the expected value for a fully hydrated peptide. The heat capacity of the native state is about 0.075 cal/K g higher than that observed for other proteins.

either one (or both) of the following possibilities. (1) The protein under consideration does not reach a completely unfolded state upon being heated; this results in a lower than theoretically predicted absolute heat capacity for the high-temperature ("denatured") state of the protein, and, consequently, in a smaller ΔC_p value. (2) The absolute heat capacity of the native state of the protein is higher than in most other proteins, and therefore, the ΔC_p value is lower than usual, even though the protein undergoes a complete thermal denaturation. In the case of the H3−H4 dimer, the first possibility should be considered nonapplicable, since our experimental data clearly show that heating of the H3−H4 dimer at low ionic strength and pH 4.5 promotes the coupled dissociation and complete unfolding of the interacting polypeptides (Figure 6). This conclusion is supported by the graphic presentation of the partial specific heat capacity function of the H3−H4 dimer at pH 4.5 and low salt (Figure 9). This figure illustrates first, at the lower side of the temperature range, a gradual increase of the heat capacity, next the transition region, and last the characteristic increase of the unfolded state at high temperatures. It is evident that the heat capacity of the high-temperature state of the H3−H4 dimer is very similar to the one expected for a completely hydrated, unstructured polypeptide of the same amino acid composition (Privalov & Makhataze, 1990; Freire, 1994; Gomez et al., 1995), suggesting that the H3−H4 dimer in this chemical environment becomes maximally unfolded and hydrated upon thermal denaturation. The experimental results also indicate that at 20 °C the heat capacity of the native state of the H3−H4 dimer is 0.425 cal/K g, a value significantly higher than that of 0.35 cal/K g reported for most globular proteins.

According to several studies (Makhatadze & Privalov, 1990; Privalov & Makhatadze, 1990; Gomez et al., 1995), three major terms contribute to the absolute heat capacity of a protein: (a) a term that depends on the primary, or covalent, structure of a protein, (b) a term containing the contributions of noncovalent interactions and, thus, representative of the secondary and tertiary structures, and (c) a hydration term. The term that deserves more careful examination in the case of the H3−H4 dimer is, by far, the hydration term, since it is the one that mostly distinguishes these data from those of other calorimetrically studied proteins, including the H2A−H2B dimer. Although the amino acid composition of the H3−H4 dimer is quite similar to that of an average soluble protein, its three-dimensional amino acid organization is quite different; the H3−H4 dimer is characterized by an unusually high apolar accessible surface area in its native state. This can be easily explained by the fact that, in its actual biological environment, the H3−H4 dimer is "shielded" from direct contact with the solvent through its extensive interactions with the other core histone proteins and with DNA, toward most possible directions. First, two H3−H4 dimers interact with each other to form a centrally located $(H3-H4)_2$ tetramer, which, in turn, is flanked by two H2A−H2B dimers. The areas of the $(H3-H4)_2$ tetramer that do not participate in the interfaces with the H2A−H2B dimers are covered by DNA, in a way that makes the H3−H4 dimer the least solvent accessible subunit of the core nucleosome. In the present study, we analyze the thermodynamic behavior of the isolated H3−H4 dimer in solution. Therefore, some of the relatively hydrophobic areas of the H3−H4 dimer, which when inside the nucleosome, are normally located in interfaces of protein−protein or protein−DNA contacts, are now exposed to solvent. This experimentally imposed excess solvation results in a higher-than-usual apolar hydration term which contributes positively to the absolute heat capacity of the native state. In addition, a higher-than-the-mean value (compared to that of globular proteins) heat capacity for the native state of the H3−H4 dimer is consistent with the NMR (Bradbury & Rattle, 1972; Bradbury et al., 1973) and crystallographic (Arents et al., 1991) findings that about 30% of all amino acids of the protein (the labile N-termini) are in random coil configuration in the native state and consequently exhibit a higher degree of hydration.

Another point of interest is the fact that the H3−H4 dimer is thermally more stable than the H2A−H2B dimer. Comparison of corresponding data for the two dimeric proteins reveals that the unfolding temperature for the H3−H4 dimer is approximately 20 °C higher than that for the H2A−H2B dimer under similar experimental conditions [56.8 vs 34.3 °C, respectively, at 75−100 mM NaCl pH 4.5)]. The enthalpy change associated with the unfolding of the H3−H4 dimer is about 10 kcal/mol of 13 kDa monomer unit smaller than the corresponding value for the H2A−H2B dimer (approximately 15 vs 25 kcal/mol of 13 kDa monomer, respectively, at 25 °C, under the aforementioned conditions). Therefore, the additional stabilization of the H3−H4 dimer, compared to the H2A−H2B dimer, necessarily results from the entropic term of the free energy function.

CONCLUSIONS

We have demonstrated here that the $(H3-H4)_2$ tetramer, when transferred to low-salt solutions of pH 4.5, is dissociated into two H3−H4 dimers. The thermal unfolding of the H3−H4 dimer is both ionic strength and protein concentration dependent and can be described as a two-state process, in which the native dimer undergoes a coupled dissociation and unfolding to two unfolded monomers, as was the case

for the H2A−H2B dimer. The higher thermal stability of the H3−H4 dimer, relative to that of the H2A−H2B dimer, is shown to be of entropic origin. Under all conditions studied, an intermediate population of folded monomer chains could not be detected. The relatively small difference between the heat capacities of the native and completely denatured states (compared to most other proteins studied thus far) can be explained by a higher-than-usual value of the heat capacity function for the native H3−H4 dimer. This results from the fact that, under the experimental conditions used, the isolated H3−H4 dimer exposes to the solvent additional hydrophobic areas, usually found at macromolecular contact interfaces within the nucleosome core, i.e., tetramer−dimer and tetramer−DNA interfaces, and thus normally shielded from the solvent.

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