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pH EFFECTS ON THE STRUCTURE OF THE INNER HISTONES

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The structure of the inner histone complex extracted from chicken erythrocyte chromatin with 2 M NaCl has been studied as a function of pH. At pH 6, the complex dissociates to (H3-H4)₂ tetramer and H2A·H2B dimer, with little change in α -helix content (as monitored by circular dichroism at 222 nm). Although the circular dichroism of tyrosyl side chains is also largely unchanged by the dissociation, measurements of intrinsic fluorescence do suggest a change in the environment of one or more tyrosines as a result of dissociation. Below pH 4, the histones become partially unfolded, lose specific secondary and tertiary structure, and undergo nonspecific aggregation. Both the pH 6 and 4 transitions, which are largely reversible, parallel pH-induced structural changes of nucleosomes (Zama, M., Olins, D.E., Prescott, B. and Thomas, G.J. (1978) *Nucleic Acids Res.* 5, 3881–3897). The results are consistent with the presence of tyrosine residues at the histone subunit-subunit contacts and suggest that histone conformation within the globular regions is largely independent of histone-DNA interactions.

Introduction

Nucleosome core particles consist of 146 bp of DNA associated with two copies of each of the four inner histones (H2A, H2B, H3 and H4) (see McGhee and Felsenfeld [1] for a review). Cross-linking studies [2] suggest that these histones form an octameric complex in chromatin, consistent with solution data showing strong pairwise interactions between the histones [3,4]. In 2 M NaCl the inner histones can form an octamer in the absence of DNA [5–11]. This unusual protein complex, consisting of two each of four nonidentical subunits, is apparently stabilized by weak in-

teractions which are easily disrupted by low salt, high temperature, low protein concentration, or extremes of pH [5,8,9]. The sensitivity of the octamer to small environmental changes may be a requirement for the control of dynamic changes in chromatin structure during transcription or replication.

In this paper we present spectroscopic studies on the acid dissociation of the histone octamer [5,9,12]. We find no major changes in secondary structure during dissociation of the octamer; however tyrosine fluorescence changes not due to simple exposure are observed.

Materials and Methods

Inner histones were prepared from chicken erythrocyte nuclei as described [8,13]. Concentrations were determined using $E_{278}^{0.1\%} = 0.454$ with the

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following spectral ratios: $A_{230}/A_{278} = 9.5 \pm 0.1$; $A_{278}/A_{260} = 2.35 \pm 0.05$. The purified histones contain < 1% contamination by any other protein, based on SDS-polyacrylamide gel electrophoresis [14].

Buffers contained 2 M NaCl and 50 mM of the desired buffer salt, as follows: pH 2–3.4, glycine; pH 3.8–5.8, acetate; pH 6.2–7.4, piperazine-*N,N'*-bis(2-ethanesulfonic acid) (Pipes); pH 7–9.4, Tris-HCl. The reported pH was determined at 22–23°C, and all experiments were at 20–23°C. The pH of each sample was determined immediately before each experiment and generally again at the end of the experiment. Measurements of pH were performed with a Corning Model 12 pH meter and a Corning semi-micro combination electrode (Cat. No. 476050).

Absorbance spectra were recorded with a Cary 15 spectrophotometer. CD spectra were obtained with a JASCO J-40A spectropolarimeter interfaced to a computer for data reduction. The instrument was calibrated with camphor sulfonic acid as described in the JASCO manual. Fluorescence measurements were made with a Perkin Elmer MPF 44A spectrofluorometer. Excitation was at 275 nm with spectral slit widths of 2 nm for both excitation and emission. As a reference to correct for possible instrument or sample fluctuations, a solution of *N*-acetyltyrosinamide was monitored before and after each sample measurement. Histone fluorescence is referred to that of free tyrosine in the same buffer and expressed as the ratio,

$$R_{\text{Tyr}} = \frac{F_{\text{Histones}}}{F_{\text{Tyr}}} \times \frac{A_{275, \text{Tyr}}}{A_{275, \text{Histones}}}$$

according to the method described by Cowgill [15].

Results

The core complex used in this work was characterized by SDS-polyacrylamide gels and ultracentrifugation at neutral pH as shown in our previous paper [8]. Quantitative scanning of the gels indicates approximately equimolar amounts ($\pm 10\%$) of H2A, H2B, H3 and H4. In our preparative sucrose gradients (at pH 7), we find a small amount of dissociated H2A and H2B, consistent with the results of others [5,9,10]. Based on

these results, our preparations appear to have a molar ratio of (H2A + H2B)/(H3 + H4) of 0.85–0.95. We believe that this represents an equilibrium mixture of (H3-H4)₂ tetramer, (H3 = H4)₂ (H2A-H2B) hexamer, and smaller amounts of (H2A-H2B) dimer and (H3-H4)₂ (H2A-H2B)₂ octamer. However, our preparation does form an octameric complex under the appropriate conditions, as shown by our results in 4 M NaCl [8], in which we found $M_w^{\text{app}} = 102000$, as expected for a mixture of $\approx 70\%$ octamer and $\approx 30\%$ hexamer (this stoichiometry is consistent with the polypeptide stoichiometry from our SDS-polyacrylamide gels).

We have confirmed (data not shown) the pattern of octamer dissociation observed previously below pH 6 [5,9,10,17]; at pH 5 we observe only the (H3-H4)₂ tetramer and (H2A-H2B) dimer in 2 M NaCl.

Circular dichroic studies of inner histone complexes

CD demonstrates that the histones contain approx. 50% α -helix in 2 M NaCl, pH 7 [18]. At this pH $[\theta]_{222} = -16.2 \cdot 10^3$ (see Fig. 1). This value is essentially constant from pH 9.5 to 6.5. However, below pH 6 there is a partial loss of α -helix, although the histones retain a helical content of 25–30% even at pH 1.4. This residual α -helix content is attributable to the stabilizing effect of salt on the histone structure [5], since the helix content increases from 0% in 1 mM HCl (pH 3) [19] to 30–35% in 2 M NaCl at pH 3.0–3.5 (Fig. 2). The midpoint of the acid transition in 2 M NaCl is near pH 4.4, well below the midpoint of the pH 6 dissociation.

Above 250 nm, the histone CD spectrum reflects contributions from aromatic side chains. The core complex spectrum (Fig. 2, inset) strongly resembles that of tyrosyl model compounds as expected from the lack of tryptophan in histones. The observed ellipticity, $[\theta]_{280} = 1.2 \cdot 10^3$, is relatively high compared to other tyrosyl-containing proteins [20,21].

Below pH 5, $[\theta]_{280}$ decreases dramatically (Fig. 2). The midpoint of this transition is pH 4.2 and suggests widespread loss of native tertiary structure. These changes occur at the same pH as the loss of α -helix shown in Fig. 1. Back titration of a histone sample from pH 3 to 7.5 results in

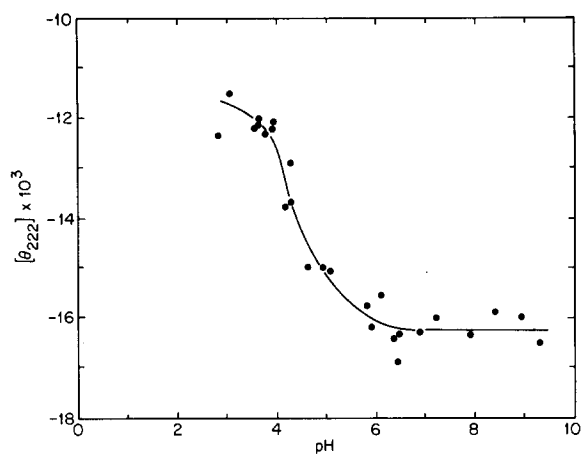


Fig. 1. Dependence of peptide CD on pH. Ultraviolet spectra were recorded, and a portion of the sample was transferred to a 0.05-cm cylindrical cuvette. CD spectra were recorded from 300–200 nm as described in the text, and the mean ellipticity was calculated. Buffer contained 2 M NaCl/0.1 mM dithiothreitol 50 mM buffer salt (pH 9.5–8, Tris-HCl, pH 7.4–6.2, Pipes; pH 5.8–4.0, acetate; pH 2.2–3.5, glycine).

recovery of 80–85 of CD intensity at 280 nm. The CD titration at 280 nm again appears to reflect histone denaturation at pH approx. 4–4.5; no change is observed at the pH of octamer dissociation.

Fluorescence spectroscopic studies of inner histone complexes

The core complex shows fluorescence typical of tyrosine [15,22] with excitation and emission maxima at 275 and 303 nm, respectively. The fluorescence intensity is constant between pH 5.5–8.5 (Fig. 3). At high pH, fluorescence is strongly

TABLE I
FLUORESCENCE CHARACTERISTICS OF INNER HISTONE COMPLEXES

pH	$R_{\text{Tyr}}(\text{H}_2\text{O})^a$	$R_{\text{Tyr}}(^2\text{H}_2\text{O})^b$	$K_Q(\text{H}_2)^c$
7.5	0.445 ± 0.02	0.582 ± 0.03	2.4 ± 0.1
4	0.55 ± 0.01	0.567 ± 0.02	2.3 ± 0.2
2.5	0.58 ± 0.02	–	1.4 ± 0.2

^a Fluorescence intensity relative to tyrosine in 100% H₂O.

^b Fluorescence relative to tyrosine in 90% ²H₂O.

^c Potassium iodide quenching constant in H₂O buffer.

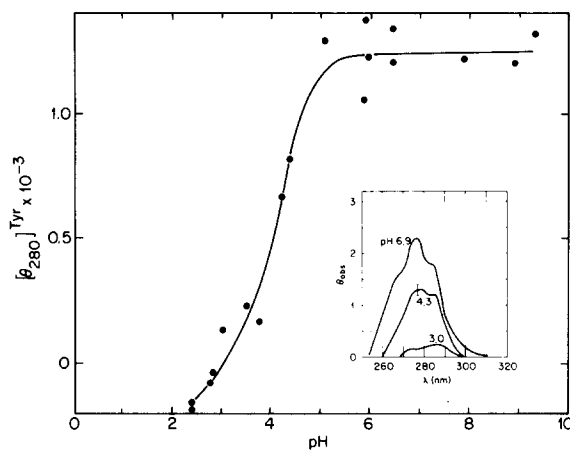


Fig. 2. Near-ultraviolet CD of inner histones. Spectra were recorded from 250–320 nm (see inset). Samples were prepared as described in the legend to Fig. 2 with the same buffer systems. Molar ellipticities were calculated based on the concentration of tyrosine residues.

quenched due to formation of the tyrosinate ion [22,23]. Between pH 5.5 and 4.5, the fluorescence intensity increases by about 25% (Fig. 3). This transition occurs at a higher pH than the two CD transitions reported above, and may result from the loss of subunit-subunit contacts when the octamer dissociates.

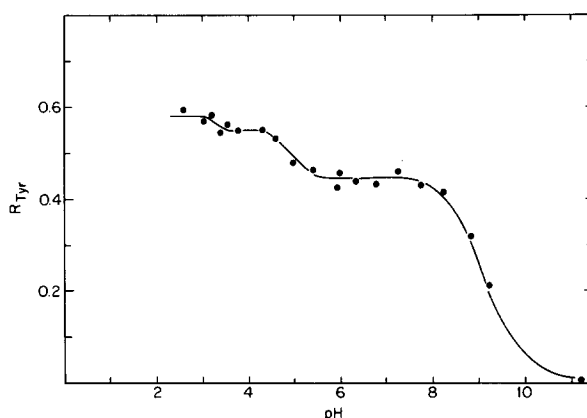


Fig. 3. Dependence of inner histone fluorescence intensity on pH. Samples were prepared as described in the legend to Fig. 2. Fluorescence intensity (at the peak, 303 nm) was corrected for a contribution from solvent Raman scattering (approx. 2% of the total intensity) and expressed relative to the intensity of L-tyrosine, as described in the text. Excitation was at 275 nm. Slit widths on both monochromators were 2 nm.

Potassium iodide quenching experiments (Table I) indicate that the average accessibility of fluorescent tyrosines to iodide is not appreciably changed over this transition. However, fluorescence spectra recorded in 90% $^2\text{H}_2\text{O}$ solutions show an increase in intensity (relative to spectra in 100% H_2O) at pH 7.5 of $\approx 30\%$, while the intensity increases only $\approx 3\%$ in $^2\text{H}_2\text{O}$ at pH 4 (Table I).

Discussion

These studies reveal that the secondary and tertiary structures of the inner histones are quite stable over a broad range of pH values. The quaternary arrangement present between pH 7 and 9.5, however, is rather delicate. The associations required for octamer stability can be disrupted by pH or decreased salt [5–10] with essentially no loss of secondary or tertiary structure (Figs. 1 and 2). This is consistent with the thermodynamic results of others [16] showing that the free energy of octamer dissociation is 7.9 kcal/mol, suggesting that only a limited number of noncovalent interactions are involved. It has been proposed [9,16] on the basis of temperature and denaturation studies that these interactions are primarily hydrogen bonds.

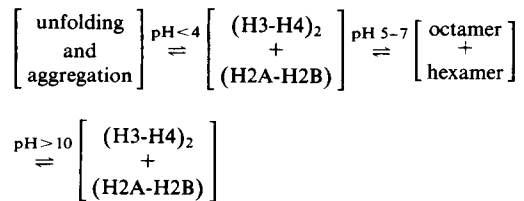
The fluorescence changes observed in the pH range (6–4.5) of octamer dissociation are indicative of alterations in the environment of at least a portion of the histone tyrosyl groups. Model studies indicate that hydrogen-bonding interactions of the tyrosyl-OH group lead to quenching [22]. Laser Raman spectroscopy [18] suggests that the majority of tyrosyl-OH groups are hydrogen bonded at neutral pH; disruption of some of these interactions may be responsible for the increase in fluorescence at pH 5. Furthermore, since $^2\text{H}_2\text{O}$ would be expected to weaken hydrogen-bonded interactions, the data in Table I are consistent with breakage of tyrosine hydrogen bonds during octamer dissociation.

Similar patterns of tyrosyl hydrogen bonding appear in nucleosomes [18]. Crosslinking of chromatin with tetranitromethane produces an H2B-H4 dimer [22]. This 'zero-length' crosslink is generated via a tyrosyl free radical. Formation of this crosslink is also sensitive to conformational changes in the nucleosome [25]. This is additional evidence

for the presence of one or more tyrosine residues at the histone subunit-subunit contacts in chromatin as well as in solution.

Although a low resolution three-dimensional density map of the octamer (based on image reconstruction of electron micrographs) has recently been published [26], structural data pertaining to the location of amino acid side chains in histones remain unavailable. A variety of approaches, including use of site-specific crosslinking agents [24,25], reconstitution of histone complexes using histone variants or chemically-modified species, and spectroscopic techniques, will be necessary to determine the types of noncovalent interactions which stabilize the octameric structure.

We can summarize the pH-dependent inner histone structural transitions (in 2 M NaCl) as follows:



The evidence for the acidic transitions has been summarized above. Eikbush and Moudrianakis [9] have shown, in addition, that the octamer dissociates to the $(\text{H3-H4})_2$ tetramer and (H2A-H2B) dimer above pH 10.

This behavior in solution appears to parallel pH-induced conformational changes which have been observed with nucleosomes [27–29]. The nucleosome conformation is stable over a pH range of 8–5.5 [27]. The histones in the core particle contain 50% α -helix [18], and the DNA CD is suppressed relative to free DNA. Between pH 5.5 and 4.8, the DNA CD becomes even more suppressed (i.e., less B-like), while no changes are observed in histone secondary structure [27]. At pH 4.6–4.2, the DNA becomes more B-like, histone α -helical character decreases slightly, and H3 thiol groups appear to move apart. Finally, at pH < 4, protonation of DNA bases and particle aggregation are observed. It is likely that the first of these nucleosome transitions corresponds to the octamer dissociation observed at about pH 6, the second to the partial histone unfolding observed

near pH 4, and the third to DNA protonation.

It is remarkable that nucleosomal DNA appears to play such a small role in the conformation of histones within chromatin, despite the relatively large interaction energies involved in histone-DNA interactions [30,31]. A number of experimental approaches have suggested that the nucleosome is organized into two distinct domains: a protein core, presumably consisting of the globular, non-polar portions of the inner histones, and a DNA-rich shell which probably also contains the highly basic histone 'tails' [1,32-34]. This DNA-rich shell appears to be responsive to changes in ionic conditions or solvent composition, whereas the protein core is more stable and may, in fact, act as a restoring force in the maintenance of nucleosomal structure [35].

In vivo modification of histones, substitution of allelic histones or of nonhistones, or binding of regulatory factors to the nucleosome could alter this balance of forces, thereby eliciting altered chromatin conformations which may be requisite for transcription and replication.

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