Studies of the DNA Binding Properties of Histone H4 Amino Terminus

THERMAL DENATURATION STUDIES REVEAL THAT ACETYULATION MARKEDLY REDUCES THE BINDING CONSTANT OF THE H4 "TAIL" TO DNA

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The effect of acetylation on the DNA binding properties of the rigidly conserved histone H4 amino-terminal tail has been studied in detail using the technique of thermal denaturation. The quantitative DNA-binding parameters for both the non- and fully acetylated H4 amino terminus have been determined from thermal denaturation data for complexes of the peptides bound to mixed sequence 146-base pair DNA. We find that under dilute buffer conditions (5 mM Tris-HCl) the binding constant for the non-acetylated peptide to double-stranded DNA is $5 \times 10^{11}$ M$^{-1}$ and that acetylation of lysine residues in the peptide reduces the binding constant to $1 \times 10^{10}$ M$^{-1}$. The dramatic differences observed in the binding constants for the non- and fully acetylated peptides are probably due to the effect of acetylation on the even distribution of positively charged residues in the H4 amino terminus. In other experiments, the binding of both peptides to a 30-base pair oligonucleotide has been studied in solution with varying concentrations of sodium, magnesium, and phosphate ions. These experiments demonstrate that both magnesium and phosphate ions have strong effects on the binding of the H4 tail to DNA, especially weakening the binding of the acetylated peptide. For instance, the dissociation of the non-acetylated peptide from DNA requires 6 mM magnesium, yet the binding of the acetylated peptide is abolished in only 30 μM magnesium. The modulation of the DNA binding interactions of the H4 amino terminus by physiologically relevant ionic conditions, in addition to the effect of acetylation, can be important in the regulation of chromatin structure and function.

The core histones are among the most highly conserved proteins found in nature. All four of the core histones (H2A, H2B, H3, and H4) have multiple domain structures, consisting of randomly coiled, very basic amino-terminal regions and globular carboxyl-terminal domains. In addition, H2A and H3 have short carboxyl-terminal tails. The tail regions of the histones constitute approximately 20% of the mass of the proteins and have been structurally defined by a combination of nuclear magnetic resonance spectroscopy (NMR) (Cary et al., 1982; Smith and Kill, 1989; Schrot et al., 1990) and protease digestion studies (Whitlock and Simpson, 1977; Bohm et al., 1981). Histones are extensively and reversibly post-translationally modified by acetylation, phosphorylation, and ubiquitination. These reversible modifications are confined to the tail regions of the proteins (Bradbury et al., 1981; Reeves, 1984). For example, the amino-terminal tail of histone H4 contains 4 lysine residues (lysines 5, 8, 12, and 16) which are in vivo sites of acetylation (acetyl sequence shown in Fig. 1). DNase I digestion and DNA/protein cross-linking studies have shown that H4 amino-terminal and other histone tails interact with nucleosomal DNA (Whitlock and Simpson, 1977; Ebralidse et al., 1988; Ausio et al., 1989). It is generally thought that acetylation of the lysine residues in the basic histone tails, which neutralizes the positive charges on the lysine epsilon amino groups, weakens and modulates the interaction between histones and DNA. However, many structural studies have shown there to be only subtle effects of acetylation on monomer nucleosome structure (Yau et al., 1982; Ausio and Van Holde, 1986; Imai et al., 1986).

There is, however, considerable interest in histone acetylation because of the strong correlation between acetylation, especially of histones H3 and H4, and gene activation. For instance, an antibody specific for the acetylated lysine side chain has been used to successfully fractionate chicken erythocyte chromatin on the basis of histone acetylation. The antibody bound, highly acetylated chromatin was found to be 15–30-fold enriched in the active β-globin gene but not enriched in the inactive ovalbumin gene (Hebbs et al., 1988). Many other techniques have been used to fractionate chromatin into "active" and "inactive" fractions, including differential salt solubility (Davie and Candico, 1978; Perry and Chalkley, 1982), mercury affinity column chromatography (Chen and Alfrey, 1987; Johnson et al., 1987), and hydroxyapatite chromatography (Imai et al., 1986). All of these methods consistently support a direct, positive correlation between histone acetylation and transcriptionally active chromatin.

Recent genetic approaches in yeast have defined new potential functions for the histone tails, especially implicating
the amino-terminal tail of histone H4. The ability to alter specific residues and regions in the amino-terminal tails of the histones using yeast genetics has been used to study specific genetic interactions between the histone tails and other protein factors involved in gene activation. This work has shown that the amino-terminal tail of H4 plays an unique role in mediating the silencing of the mating type loci in Saccharomyces cerevisiae. The very basic sequence Lys-Arg-His-Arg (residues 16–19 of H4) is required for the repression of the mating type loci, through direct or indirect interactions between this region of H4 and yeast regulatory proteins (Kayne et al., 1988; Johnson et al., 1990). In addition, deletion of the H4 amino-terminal tail represses the activation of GAL1 and PHO5 promoters in S. cerevisiae (Durrin et al., 1991). Both of these important functions are unique to the amino-terminal tail of histone H4 and are not shared with the other three core histones.

In this paper, we use thermal denaturation to study the binding interactions of both non- and fully acetylated H4 amino-terminal tail peptides to DNA under varying ionic conditions. It is shown that acetylation of lysine residues in the H4 amino-terminal tail markedly decreases its affinity for non-purified nucleosomal length DNA, reducing the binding constant by six orders of magnitude in dilute buffer. We suggest that the large differences observed in the binding constants of the two peptides is probably due to the distribution of positively charged residues in both non- and fully acetylated peptides. We also show that the binding of the two peptides (non- and fully acetylated) to a 30-bp DNA oligonucleotide is differentially dependent upon the solution conditions used and that the binding of the acetylated peptide is especially sensitive to both magnesium and phosphate ions. Our results quantitatively demonstrate the strong effect that histone acetylation can have on histone-DNA interactions and also suggest possible specific interactions between acetylated chromatin and cations.

**Materials and Methods**

**Preparation of Non- and Acetylated H4(1–23) Peptide—**A mixture of histone H4/H2A (fraction F2 as described by Johns, 1967), at 10 mg/ml was hydrolyzed in 0.25 M acetic acid, under vacuum, at 105 °C for 24 h (Cary et al. 1982). The hydrolysate was lyophilized and dissolved in deionized distilled H2O at a concentration of 10 mg/ml. The H4(1–23) peptide was purified from the hydrolysate by C-4 reverse-phase HPLC column chromatography (Phenomenex W-Porex 5 C4, 250 × 10 mm). The column was eluted with a linear gradient of 6–14% acetonitrile (0.1% trifluoroacetic acid) over 90 min. The non-acetylated peptide eluted as a single peak (Fig. 2), which was pooled and lyophilized, and finally redissolved in deionized distilled H2O. The mono-acetylated peptide peak was pooled, lyophilized, dissolved in water, and used to prepare the chemically acetylated peptide. Chemical acetylation of the H4(1–23) peptide with acetic anhydride was done on ice in 100 mM sodium boronate at a peptide concentration of 2 mg/ml (Cary et al., 1982). A 100:1 molar ratio of acetic anhydride/lysine residues was added, with stirring, in small aliquots over 30 min. The pH of the acetylation reaction was maintained at about 9.0 by the addition of NaOH. The acetylated peptide was purified from the reaction mixture by HPLC, lyophilized, dissolved in deionized distilled H2O, and the purity checked by acid-urea gel electrophoresis (Fig. 3). The concentrations of both non- and acetylated peptide were determined by amino acid analysis on a Beckman 6300 amino acid analyzer to an accuracy of ±5%

**Acid-Urea Gel Electrophoresis of Peptides—**The H4(1–23) peptides were analyzed on 20% acid-urea polyacrylamide gels, 350:2, acrylamide to bis-acrylamide ratio; containing 2.5 M urea and 5% acetic acid; gel size: 20 cm x 20 cm x 0.75 mm. The gels were prerun at 150 V for 5 h or until the conductivity became constant. After prerunning, the reservoir buffer (5% acetic acid) was poured off and replaced with fresh buffer. Gels were run at 250 V for about 2.5 h or until the green component of methylene green dye reached about two-thirds of the gel length. Gels were stained in 0.1% Amido Black, 40% methanol, 10% acetic acid, and destained in several changes of 10% acetic acid, 1% ethanol.

**Preparation of DNA—**Mixed sequence DNA (about 146 bp) was prepared from nucleosome core particles (Wood et al., 1991). After the histones were extracted with phenol and chloroform, the nucleosomal DNA was precipitated with ethanol, washed in 80% ethanol, dried, and redissolved in TE buffer (10 mM Tris·HCl, pH 7.5, 1 mM EDTA). The 146-bp DNA was then dialyzed extensively against the experimental buffer 5 mM Tris·HCl, pH 7.5.

A 30-bp synthetic DNA was synthesized on an Applied Biosystems DNA synthesizer. The oligonucleotide was designed to be a random sequence containing equal amounts of A/T and G/C base pairs. The two strands of the oligonucleotide were purified on reverse-phase C-4 HPLC column, buffered with 0.1 M TEAA (triethanolammonium acetate), using a 0–50% acetonitrile gradient over 50 min. Duplex DNA was prepared by annealing the two single-stranded oligonucleotides in 30 mM NaCl at a concentration of 4 mg/ml.

**Thermal Denaturation Studies—**Thermal denaturation experiments were performed on a Cary 210 spectrophotometer in double beam mode using a 10-mm pathlength sealed quartz cuvette (Yau et al., 1982). The temperature was controlled by a Neslab water bath and temperature programmer, with a heating rate of 0.5 °C/min. Data collection, filtering, and differentiation were performed on a Hewlett-Packard 286 computer interfaced to the Cary 210 spectrophotometer. The binding constants were calculated as described under “Appendix.” Prior to each experiment the samples were mixed thoroughly and degassed. The final DNA concentrations used in the experiments were 18.3 µg/ml for the 146-bp DNA and 20 µg/ml for the 30-bp DNA. The peptide concentrations are given in the figures for each experiment.

**Results**

**Thermal Denaturation Studies of H4(1–23)-DNA Complexes**

We have studied the effect of binding of the amino-terminal, 23-amino-acid peptide of histone H4 on the melting curve of DNA and have extensively compared the binding of both non- and fully acetylated peptides at several different peptide/DNA ratios under various ionic conditions. The melting data of both 146-bp mixed sequence DNA (derived from nucleosome core particles), and a 30-bp synthetic DNA oligonucleotide have been used in these studies. The 146-bp DNA was prepared as described (Kayne et al., 1988; Johnson et al., 1991) in very low salt, where we have varied the peptide to DNA ratios. These experiments allow for the determination of binding constants for both the control and acetylated peptides. The 30-bp DNA was used for experiments involving higher salt, since this DNA gives a reasonable melting temperature under all of the experimental conditions, i.e. it melts at temperatures between 53 and 75 °C over a wide range of ionic strengths. Longer fragments have very high melting temperatures in high ionic strengths and are therefore difficult to use in these types of studies. Because the binding of this histone peptide to DNA is not expected to have any strong sequence specificity, the sequence of the 30-bp fragment was randomly generated with the only constraint being that it has equal proportions of A:T and G:C base pairs. The sequence of the 30-bp fragment used in these experiments is 5′-GCATGCTAATTCCGTTGTCAGCGATCTCGA-3′ and 3′-CGTACGATTTAAAGGCAACGTCGCTAGACCT-5′.

Most of our studies were carried out in 10 mM Tris·HCl buffer, pH 7.5. This buffer has been used because it is effective as phosphate or carbonate have the potential to interact directly with arginine residues through ionic and hydrogen bonding as we show later and therefore reduce the peptide/DNA binding. We anticipated a change of buffer pH with temperature and measured the pH change in the range of thermal denaturation temperatures used with our buffer system (5 mM Tris) and found that the maximum pH change in the entire
Acid urea gel electrophoresis of the fractions through the peaks of non-acetylated and mono-acetylated H4(1–23) peptides, following HPLC purification from the acetic acid digestion of the H4/H2A mixture. The C-4 reverse HPLC column (250 × 10 mm) was loaded with 10 mg of the digestion mixture and eluted with a gradient of 6–14% acetonitrile (0.1% trifluoroacetic acid) over 90 min. The retention times of the non- and mono-acetylated peptide are around 25 and 45 min.

Studies of DNA Binding Properties

The quantitative analysis of the data requires that the scan rate is sufficiently slow to allow for complete equilibration during the thermal denaturation process. We have repeated the thermal denaturation experiments on the 146-bp DNA complexes with a scan rate of 0.05 °C/min. The denaturation curves obtained are identical to those obtained at a scan rate of 0.5 °C/min, the scan rate used in our experiments. Thus, our scan rate is slow enough to achieve complete equilibration during the thermal denaturation process.

Studies of H4(1–23)/146-bp DNA Complexes Binding Constant Determination—The binding of both non-acetylated and fully acetylated H4(1–23) peptides to 146-bp DNA was studied in 5 mM Tris-HCl at pH 7.5. The 146-bp DNA was purified from chicken erythrocyte nucleosome core particles, and therefore is of mixed sequence. Fig. 4 shows the melting curves for H4(1–23)-146-bp DNA complexes under these conditions. In these experiments, the mass ratio of peptide to DNA is varied from 0 to 0.4 for the non-acetylated and from 0 to 1.1 for the acetylated peptide. As the melting data shows, at any given peptide/DNA ratio, the non-acetylated peptide stabilizes the melting of the DNA to a much greater extent, indicating stronger binding. This is expected, since acetylation of the peptide abolishes the charges on all 4 of the lysine residues in the peptide. At low peptide/DNA ratios, both peptides broaden the melting curve of the 146-bp DNA relative to that of DNA alone. Broadening of the melting transition is a typical phenomenon of nonspecific, ionic ligand-DNA interactions (Dove and Davidson, 1962; Morgan et al., 1986).

From the data shown in Fig. 4, the binding constants for both control and acetylated peptides to 146-bp DNA have been calculated using the excluded sites model developed by McGhee and von Hippel (McGhee and von Hippel, 1974; McGhee, 1976). The broadening of the melting curves due to peptide binding were calculated as a function of binding constants for double- and single-stranded DNA ($K_a$, $K_c$), size of binding site for both forms of DNA ($n_a$, $n_c$) (number of base pairs for $n_a$ or number of bases for $n_c$ covered by the binding of one peptide), $T_m$, total DNA concentration, and total peptide concentration. Binding parameters $K_a$, $K_c$, $n_a$, and $n_c$ were obtained numerically by varying their values until the best fit between the experimental and the calculated melting curves at different peptide concentrations was achieved, i.e. the total fitting error reaches its minimum, and any change in any of the four parameters would increase the fitting error (Morgan et al., 1986) (explained in detail under "Appendix"). The results are listed in Table I. The binding constant calculated for the control peptide to double-stranded DNA is $5.0 \times 10^{14} \text{ M}^{-1}$, and acetylation reduces the binding constant by a factor of 10. The sizes of the binding sites are also considerably reduced by acetylation for both double- and single-stranded DNA. The value of $K_a/K_c$, which is an indi-

\[ \text{non-acetylated H4(1-23)} \]

\[ \text{tetra-acetylated H4(1-23)} \]

\[ \text{acetic acid digestion of H4/H2A mixture} \]

\[ \text{non-acetylated H4(1-23)} \]

\[ \text{mono-acetylated H4(1-23)} \]
Studies of DNA Binding Properties

Salt Effects on H4(1-23) Binding to DNA—Melting experiments have been carried out on complexes formed between the control and acetylated H4 tail peptides and a synthetic 30-bp length DNA (sequence shown above) in 5 mM Tris-HCl, pH 7.5, in which we varied the concentration of sodium chloride. In all of these experiments the DNA concentrations were held constant at 20 μg/ml and the peptide/DNA mass ratios were 0.5:1. Fig. 5 shows melting curves comparing free DNA to both types of peptide-DNA complexes, in concentrations of 2, 10, 50, 75, 100, and 150 mM sodium chloride. At NaCl concentrations of less than 50 mM, both peptides stabilize the 30-bp DNA fragment from melting indicating that under these conditions both acetylated and control peptides are bound to the DNA. At 75 mM it appears that the acetylated peptide is no longer bound, since the addition of the peptide to the DNA does not alter the melting curve of the DNA alone under these conditions. The control peptide is still bound at 125 mM NaCl, and the binding of this peptide to DNA is apparently abolished between 125 and 150 mM NaCl.

The melting data shown in Fig. 5 is summarized in Table II, which gives the transition temperatures for each of the peaks. In addition, this table gives the differences between the melting transition for the DNA and the peptide-DNA complexes. This difference represents the stabilization given to DNA by the H4 tail peptides. At 2 mM NaCl the non-acetylated peptide stabilizes the melting of the 30-bp DNA by 16 °C, whereas the acetylated peptide only stabilizes the melting by 5 °C. At the higher concentrations of NaCl, the differences between the free DNA and the peptide-DNA complexes are not as large, since the addition of salt weakens the peptide-DNA binding interactions and also stabilizes the DNA duplex.

![Fig. 4. Thermal denaturation of non-acetylated (A) and tetra-acetylated (B) H4(1-23)-146-bp DNA complex at different peptide/DNA mass ratios. DNA concentration was kept constant at 18.3 μg/ml. Legends of the peptide concentrations: (for A and B, respectively) free DNA (—–), 1.7 and 5.2 μg/ml (— – – –), 3.3 and 8.7 μg/ml (——), 4.9 and 12.2 μg/ml (-----), 6.5 and 15.7 μg/ml (…….), 8.2 and 20.9 μg/ml (--------).](image1)

![Fig. 5. Thermal denaturation of 30-bp DNA and its complex with non-acetylated and tetra-acetylated H4(1-23) in different NaCl concentrations: 2 mM (A), 10 mM (B), 50 mM (C), 75 mM (D), 100 mM (E), 150 mM (F). Peptide/DNA = 0.5:1 (mass ratio). The molar concentrations of both the peptide and the DNA were kept constant through the experiment. ——, free DNA; —, non-acetylated H4(1-23)-DNA complex; — — —, acetylated H4(1-23)-DNA complex.](image2)

**Table I**

<table>
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<tr>
<th>Peptide*</th>
<th>logKₐ</th>
<th>logKₓ</th>
<th>nₓ</th>
<th>nₚ</th>
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<tr>
<td>Non-acetylated</td>
<td>11.7 ± 0.6</td>
<td>10.0 ± 0.5</td>
<td>17 ± 2</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>Acetylated</td>
<td>5.0 ± 0.2</td>
<td>4.0 ± 0.2</td>
<td>8 ± 3</td>
<td>1</td>
</tr>
</tbody>
</table>

*The small binding site size for the acetylated peptide binding to single-stranded DNA probably reflects the absence of tightly localized binding.

**Table II**

<table>
<thead>
<tr>
<th>[NaCl] (mM)</th>
<th>T₁,1 (°C)</th>
<th>T₂,1 (°C)</th>
<th>T₁,2 (°C)</th>
<th>T₂,2 (°C)</th>
<th>T₁,3 (°C)</th>
</tr>
</thead>
<tbody>
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<tr>
<td>150</td>
<td>74</td>
<td>74</td>
<td>74</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*T₁,1, transition temperatures of DNA.
*T₂,2, transition temperatures of DNA-non-acetylated H4(1-23) complex.
*T₁,3, transition temperatures of DNA-acetylated H4(1-23) complex.
Yet even at 100 mM NaCl, where the difference between free DNA and control peptide-DNA complex is only 1°C, the curves are clearly different (Fig. 5) and are indicative of binding. These results are consistent with the binding constants we have determined for these peptides on 146-bp DNA (see "Appendix"), which indicates that acetylation of the H4 amino-terminal tail peptide dramatically lowers the binding of the peptide to DNA.

We have also investigated the effect of divalent magnesium ions on the binding of the two peptides to the 30-bp synthetic DNA molecule. Fig. 6 shows the results of thermal denaturation experiments determined for the complexes in the presence of varying concentrations of magnesium chloride. The conditions of these experiments were the same as used in the experiments on the effect of NaCl (i.e. 20 μg/ml DNA in 5 mM Tris-HCl, pH 7.5). The MgCl₂ concentrations used in these experiments are 0.005, 0.015, 0.03, 1, 6, and 15 mM. Again under these solution conditions the control peptide stabilizes the DNA from melting considerably more than the acetylated peptide, indicating its stronger binding to DNA. However, we find striking differences in the ability of the two peptides to stabilize the 30-bp DNA fragment from melting in the presence of magnesium ions. The acetylated peptide binds very weakly even in 5 mM MgCl₂, stabilizing the DNA by only 2°C, and its binding is completely abolished between 15 and 30 μM MgCl₂. In contrast, the control peptide binds well to the DNA in the presence of low concentrations of MgCl₂, since the melting transition for the 30-bp DNA in 5 and 15 mM MgCl₂ is increased by 13 and 11°C, respectively. In addition, the peak broadening seen in these complexes is as significant as was seen in low concentrations of NaCl, which also indicates strong interactions. The binding of the control peptide is not completely abolished until the MgCl₂ concentration is raised to 6 mM. These results are summarized in Table III.

The relative effect of the two types of salts, NaCl and MgCl₂, on the binding of the H4 amino-terminal peptides to the 30-bp DNA is quite marked. In solutions containing either sodium or magnesium chloride, the binding of the nonacetylated peptide is always significantly stronger, consistent with its much stronger binding constant in dilute buffer. However, whereas about two-thirds of the amount of NaCl (75 versus 125 mM) is required to eliminate acetylated peptide binding to DNA, compared with the non-acetylated peptide, only about 1/200 the concentration of MgCl₂ has the same effect (0.03 versus 6 mM). This indicates that the binding of the acetylated peptide is especially sensitive to the presence of even small amounts of magnesium cations. In addition, the concentration of MgCl₂ required to eliminate the binding of the nonacetylated peptide to DNA is about 1/20 of the concentration of NaCl required to have the same effect (6 versus 125 mM). In contrast, the concentration of MgCl₂ required to eliminate binding of the acetylated peptide to DNA is only 1/2500 of the concentration of NaCl necessary to observe the same effect (0.03 versus 75 mM).

Effect of Phosphate Buffers on H4(1-23)-DNA Interactions—Many experiments on the binding of proteins to DNA have been performed in phosphate buffers. Early NMR studies on the interaction of amino-terminal tail peptides of H4 with DNA were carried out in phosphate buffers (Cary et al., 1982), and these studies concluded that acetylation completely abolishes the binding of the H4(1-23) peptide, even in very low ionic strength conditions (10 mM phosphate buffer only, no additional salt). Our results indicate that acetylation, while markedly reducing the affinity of the peptide to DNA, does not abolish the binding in low ionic strengths (see Figs. 4–6). In an effort to reconcile these two results, we have studied the binding of the H4(1-23) peptides to DNA in solutions containing phosphate buffers, instead of the Tris-HCl buffers used in the experiments described above.

Fig. 7 shows the melting curves for both acetylated and control peptide-DNA complexes studied in either: (A) 5 mM Tris-HCl, pH 7.5, 50 mM NaCl, or (B) 5 mM sodium phosphate, pH 7.5, 41 mM NaCl. In both buffers the total Na⁺ ion concentration is 50 mM. We have taken into account the amount of NaOH used to titrate the phosphate buffer to the desired pH. In both of these buffers the melting profiles of the free DNA are identical, indicating that the buffers alone do not have differential effects on the melting of the DNA, and also that the Na⁺ ion concentrations in the two solutions are indeed the same. In Tris buffers, both the acetylated and
nonacetylated peptides stabilize the melting transition of the DNA duplex (Fig. 7A). However, in phosphate buffer, no apparent binding is observed for the acetylated peptide, yet the nonacetylated peptide still interacts with the DNA (Fig. 7B). Also, we find that the $T_m$ for the non-acetylated peptide-DNA complex is considerably lower in phosphate buffer than in Tris buffer, indicating weaker binding. Therefore, the binding of both peptides to the DNA is obviously weakened by phosphate buffer compared to Tris buffer.

These results show that the above melting data on the H4(1-23)-DNA complex are biochemically consistent with the early NMR data on the same complex. Acetylation of the H4(1-23) tail does not neutralize all of the positive charges on the peptide, which still retains 4 cationic arginine residues, and would be expected to interact with DNA. For instance, it has been shown that the dipeptide Arg-Arg binds three times stronger to DNA than a Lys-Lys dipeptide (Helene and Maurizot, 1981). These differences in binding are presumably due to the types of interactions observed between lysine and arginine residues and DNA. The guanidinium group of the arginine side chain has the potential for both ionic and hydrogen bonding interactions with the phosphate backbone of the DNA, while the interactions of the amino group of the lysine side chain with DNA are usually simple ionic (Helene and Maurizot, 1981). Because of this one would also expect the guanidinium group of arginine to bind strongly to inorganic phosphate ions through both ionic and hydrogen bonding interactions. It appears that in this case, the phosphate buffer effectively neutralizes the interactions between the 4 arginine residues of the peptide and the phosphate backbone of the DNA, thereby weakening the binding of both peptides, and completely abolishing the binding of the acetylated peptide. Once the charges on the lysine residues of the acetylated peptide are neutralized by acetylation, and the arginine residues are effectively neutralized by ion-pairing interactions with the phosphate ions of the buffer, the peptide has no cationic groups left to interact with the DNA. The results of the interactions between the peptide and the phosphates both in solution and on the DNA backbone, are that, under these conditions, the binding of the acetylated peptide is apparently abolished.

**DISCUSSION**

Acetylation is one of the major reversible histone modifications and occurs exclusively in the flexible, randomly coiled histone amino-terminal tails. The histone tails are enriched in the basic residues, lysine and arginine, which are positively charged at physiological pH, and therefore have the potential to bind to the negatively charged phosphates on the DNA backbone. The interaction of the histone tails with DNA, and the modulation of the interaction by acetylation of lysine residues in the tails, is thought to play an important role in the regulation of chromatin function. Histone acetylation correlates strongly with many DNA processing events, including transcription (Allegra et al., 1987; Zhang and Nelson, 1988), replication (Chahal et al., 1980; Waterborg and Matthews, 1984), and spermiogenesis (Christensen et al., 1984; Gatewood et al., 1988). In addition, histone tails are probably involved in the formation of higher order chromatin structures. Deletion of the histone tails in yeast has specific effects on the mating type loci, expression of certain genes, and timing of the cell cycle (Johnson et al., 1990; Durrin et al., 1991; Morgan et al., 1991). Work from our laboratory has also shown that acetylation of histones H3 and H4 reduces the negative linking number change of topologically constrained mini-chromosomes (Norton et al., 1989, 1990), and further studies have indicated that the reduction of the linking number change results solely from the contribution of histone H4 acetylation, results which suggest many other possible roles and mechanisms for histone acetylation in regulating chromatin structure and function. The global regulation of histone-DNA interactions by reversible acetylation most likely plays an important structural, and perhaps regulatory, role in a wide range of DNA processing events. Therefore, our understanding of the molecular interactions between histone tails and DNA are undoubtedly important for determining the biological effects of acetylation.

The original work on histone amino-terminal tail-DNA interactions with NMR demonstrated that the tails bound to DNA through the basic residues, lysine and arginine (Cary et al., 1982). It was also shown by NMR that the amino-terminal tails appear to dissociate from the DNA in nucleosome core particles between 0.3 and 0.6 M NaCl. However, using NMR it is difficult to quantitatively study binding (i.e. to determine a binding constant) for the tail peptides to DNA. In this study, using the technique of thermal denaturation, we are able to quantitatively study the binding of the H4 tail to DNA. In addition, we are able to study the interactions over a range of solution conditions, including some which are difficult to use in NMR experiments. By studying the effects of peptide binding on the thermal denaturation of DNA, we are able to calculate a binding constant for both non- and fully acetylated peptides in dilute buffer and demonstrate specific effects of magnesium and phosphate ions on acetylated peptide binding (see “Results”).

Early thermal denaturation studies on histone-DNA interactions were carried out on nucleosome monomers, dimers, and trimers (Yau et al., 1982; Ausio et al., 1989). The nucleosome has a molecular mass of close to 200 kDa, and its structure involves multiple domain interactions between the DNA and the histone octamer. Because of its complexity, its thermal denaturation transitions are very broad and include more than one phase. There is no single transition that is indicative of the interactions of the amino-terminal domains with the DNA. At high ionic strengths, the melting temperature is too high to conveniently measure, and the melting profile is thus less informative. Therefore, it is difficult to make a clear and quantitative evaluation of these interactions as well as the effects of acetylation under different conditions when working with nucleosomes.

In this study, we have purified the H4(1-23) amino-terminal domain from calf thymus and have examined its interactions with defined lengths of DNA. Single thermal transitions were obtained for the peptide-DNA complex under various buffer conditions. This allowed a simpler, more quantitative method to analyze the effects of acetylation on the H4(1-23)-DNA interaction. H4(1-23) contains 5 lysines, 4 of which can be acetylated in vivo (lysines 5, 8, 12, and 16; lysine 20 is methylated in vivo). All of the 4 lysines can be acetylated in vitro by treatment with acetic anhydride. The in vitro acetylated H4(1-23) is identical to the fully acetylated H4 amino-terminal domain found in vivo.

The H4(1-23) peptide has multiple positive charges which can interact with DNA in a cooperative manner. During the melting process, both double-stranded and single-stranded DNA are present. Because the double-stranded DNA has a higher negative charge density which better facilitates the cooperative ionic interaction of the multiple positive charges on each peptide with DNA, the peptide binding constant to the double-stranded DNA ($K_d$) is higher than that to the single-stranded DNA ($K_s$). Therefore, during the melting transition, peptide molecules can move from the denatured...
single-stranded DNA to the remaining double-stranded DNA which gives further stabilization to the remaining undenatured DNA. This has the effect of broadening the melting transition (Dove and Davidson, 1962; Morgan et al., 1986).

As the peptide concentration is increased, DNA is saturated to a greater degree, which leads to less peptide relocation and peak broadening is minimized.

As the ionic strength is increased, both $K_{\text{d}}$ and $K_{\text{r}}$ are reduced. Peptide binding preferentially to either the double- or single-stranded DNA would have opposite effects on the $T_m$, either increasing or decreasing it, respectively. The results show that as the ionic strength was increased, the effect of the peptide on the $T_m$ was always decreased. At the ionic strength at which the peptide no longer affects $T_m$, there are two possible scenarios. One is that the preferential decrease of $K_{\text{r}}$ over $K_{\text{d}}$ balances the stabilization - destabilization effects such that the $T_m$ remains unchanged. This is very unlikely since a further decrease in $K_{\text{r}}$ due to increased ionic strength would make the $T_m$ lower than that of the free DNA, and the peptide would become a helix destabilizer. This was never observed and therefore the more reasonable explanation is that at higher ionic strengths, peptide binding to both forms of DNA are completely abolished.

Early NMR studies indicated that acetylation totally abolished the binding of the H4 amino-terminal domain to DNA even at low ionic strengths (Cary et al., 1982). In contrast, our data indicate that there is binding of fully acetylated peptide below 50mM NaCl. Based on the amino acid composition of this peptide, the binding is most likely to be due to non-specific electrostatic interactions between the basic residues of the peptide and the DNA phosphate backbone. This interaction should be reduced rather than abolished by acetylation, since there are still 4 positively charged arginine residues present on the fully acetylated peptide at physiological pH. The discrepancy between these results and the previous NMR studies is related to the phosphate buffer effects on acetylated peptide binding, which were studied in Fig. 7. These results clearly showed that phosphate buffer lowers the affinity for both non- and fully acetylated peptides to DNA, probably by ion-pairing interactions with the 4 arginine residues in the peptide.

It should also be noted that in the non-acetylated state, the positive charges are quite uniformly distributed over the peptide (positions 3, 5, 8, 12, 16, 17, 19, and 23). This even charge distribution (positions 3, 17, 19, and 23 are arginine). Both factors most likely contribute to the reduction of peptide-DNA binding. This view is supported by the following experimental observations: (i) in 2 and 10 mM NaCl, non-acetylated peptide increased the $T_m$ by 16 and 13 degrees, but acetylated peptide only increased $T_m$ by 5 and 3 degrees, respectively. At low ionic strength (5mM Tris-HCl), the $K_{d}$ of the former is more than 10⁶ times higher than that of the latter. (ii) This is even more obvious in 5 and 15µM MgCl₂, where the $T_m$ increases were 13 and 11 degrees for non-acetylated peptide versus 3 and 1 degrees for acetylated peptide. The latter are only one-fourth and one-tenth of the former. (iii) 30µM MgCl₂ can completely suppress the binding of acetylated peptide to DNA, but more than 6mM is needed to do the same with the non-acetylated peptide, a difference of 200 times. (iv) When three times the amount of acetylated peptide was used to increase the charge ratio of peptide to DNA to more than that of the non-acetylated peptide, the $T_m$ increase for the acetylated peptide was still significantly lower than for the non-acetylated peptide (Fig. 4). (v) Acetylation reduces the number of the positive charges by 4, but the peptide-binding sites are reduced by 9 and 12 bp, respectively, for double- and single-stranded DNA. From these observations, it is clear that although the positive charge on the peptide is reduced by half by acetylation, the binding affinity is reduced by a much greater proportion. Alteration of the charge distribution on the H4 amino-terminal domain appears to be a significant factor in this reduction. It should be noted that since the peptide is relatively short (23 residues) and highly charged, we do not anticipate any secondary structure motifs such as α-helix or β-sheet. CD and NMR studies have indicated that this is true (Cary et al. 1982).² We do not think there is any effect on peptide-DNA interaction due to peptide conformation change in the process of thermal denaturation.

Interestingly, Mg²⁺ is much more effective than Na⁺ in the suppression of acetylated peptide binding to DNA. Fig. 5 shows that only 30µM Mg²⁺ is required to eliminate acetylated peptide binding to DNA, while more than 50mM Na⁺ is needed to give the same effect. The DNA concentration in the experimental samples was 20µg/ml, which is about 30µM in nucleotide base pairs. This implies that one Mg²⁺ can render the DNA base pair inaccessible to the acetylated peptide. This is not true for the non-acetylated peptide whose binding is suppressed only by greater than 6mM Mg²⁺. Again, the differences in binding constants, which is probably related to the distribution of charged residues on the two peptides, appears to be the significant factor for this phenomenon. This is a very intriguing result, since these values are in the physiological range of the ion concentrations found in eukaryotic cells. Based upon our results, it is possible that small, micromolar changes in magnesium ion concentrations could have differential effects on the stability of acetylated chromatin when compared to non-acetylated chromatin. The observed differences between the binding of acetylated and non-acetylated H4 tails to DNA under these ionic conditions could be related to the long standing observation that highly acetylated chromatin is differentially soluble in millimolar concentrations of magnesium ions.

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APPENDIX

Determination of Binding Parameters—Binding parameters can be derived from thermal denaturation data by a variety of methods. Many of these methods assume no binding to single-stranded DNA and no overlap of binding site. Both of these assumptions are invalid in the cases considered here. In particular, the assumption of non-overlapping binding sites is replaced by an assumption of sequence-independent binding with a fixed size of binding site. The problem of overlapping binding sites is easily seen by considering the example of a DNA molecule N bp long and ligand that covers n bases. The first molecule of ligand that binds “sees” N different binding sites (ignoring end effects). Once it is bound, however, there are only N-2n sites available for the second molecule of ligand (n bases are covered by the first ligand and a further n sites are unavailable because the second ligand cannot overlap the first). Binding of the second molecule of ligand will remove between n and 2n possible binding sites, depending on where it binds in relation to the first molecule. This situation of overlapping binding sites, has been treated statistically by McGhee and von Hippel (1974). McGhee (1976) showed an application of the approach to the thermal denaturation of netropsin bound to poly(dAT). This was a simplified example.
since netropsin does not bind to random coil (single-stranded) DNA. More recently, Morgan et al. (1986) extended the analysis to ligands that bind both single- and double-stranded DNA and used it to determine binding parameters for polyanines and DNA.

The procedure depends on making measurements of the melting temperature of independently melting segments of DNA as a function of ligand concentration. The data do not resolve independently melting segments, but measurements on given independently melting DNA sequences can be made by taking measurements of thermal denaturation temperature at fixed values of the hyperchromicity. We assumed that the peptide binding is independent of DNA sequence and thus that each independently melting sequence always melts in the same order in the whole thermal denaturation curve, the order being independent of the ligand concentration. Thus, at any ligand concentration and at any particular value of the hyperchromicity, the same sequence is melting as at any other ligand concentrations and the same hyperchromicity.

**Theory**—First, we made the assumption that the increase in melting temperature, $\Delta T$, due to peptide binding is function only of the occupancy of peptide-binding sites on helical DNA, i.e. the thermal denaturation temperature, $T_i$, of an independently melting sequence is given by

$$T_i = T_{i,0} + \Delta T$$

(Eq. 1)

where $T_{i,0}$ is the thermal denaturation temperature in the absence of ligand and

$$\Delta T = g(v_H)$$

(Eq. 2)

where $(g(\ldots))$ is an unknown function. Implicit in this assumption is that the form of $g(\ldots)$ is independent of both temperature and the DNA sequence which further implies that there is no conformational change of the peptide over the temperature range of the experiment.

Second, we made the assumption that the occupancy of peptide-binding sites (binding density) on helical DNA ($v_H$) is a function only of the binding parameters ($K_H$, $K_c$, $n_H$, and $n_c$), the total ligand concentration, $L_T$, and the concentrations of helical and random coil DNA ($P_H$, $P_C$), i.e.

$$v_H = f(K_H, K_c, n_H, n_c, L_T, P_H, P_C)$$

(Eq. 3)

where $f(\ldots)$ is a function that can be evaluated numerically (McGhee, 1976; Morgan et al., 1986) and thus used to calculate $v_H$ from the other parameters. Combining the last two equations shows the $\Delta T$ is a function of just the binding parameters and the known quantities $L_T$, $P_H$, and $P_C$, although we do not know the form of the function $f(\ldots)$ is known but $g(\ldots)$ is unknown.

The function, $g(\ldots)$, was determined empirically using a subset of the data. We took the thermal denaturation temperature at 50% hyperchromicity, $T_{50,0}$, at each ligand concentration and subtracted the thermal denaturation temperature in the absence of ligand, $T_{50,0}$, to give the measured $\Delta T$ values at 50% hyperchromicity. The occupancies, $v_H$, were calculated using the current estimate of the binding parameters, and the curve of $\Delta T$ as a function of $v_H$ was plotted and used as a calibration curve for empirical determination of $g(\ldots)$.

For a given ligand concentration, the $\Delta T$ at 20% hyperchromicity (for example) will be quite different from the $\Delta T$ at 50% hyperchromicity, depending on the binding parameters. This is because, during melting, random coil DNA is formed and ligand migrates from the low affinity sites on random coil DNA to the remaining high affinity sites on helical DNA, thus increasing the occupancy of sites on helical DNA and hence its melting temperature. Thus, the melting curve broadens.

We predicted the thermal denaturation temperature at 20 and 80% hyperchromicity using (i) the current estimate of the binding parameters to give $v_H$, (ii) the thermal denaturation temperatures at 50% hyperchromicity to give $g(v_H)$, and (iii) the melting temperatures in the absence of ligand, the $T_{50,0}$ values. The predictions were compared with the experimentally determined values. The calculations were repeated with different estimates of the binding parameters (and the corresponding predicted $g(\ldots)$) until we determined the set of binding parameters that gave the least square deviations of the predicted $\Delta T$ values from the experimental values.

**Calculations**—First, it was necessary to develop a procedure to calculate the binding densities on the two polymers (double helical DNA and random coil DNA) from the binding parameters and the total concentrations of the polymers and ligand. Following Morgan et al. (1986), equations were derived to relate the binding density on both random coil and double helical DNA to the binding parameters, given the total ligand concentration, the DNA concentration, and the hyperchromicity. It was assumed that the binding is not cooperative. The necessary quantities are defined in Table IV.

The first four quantities of Table IV, $K_H$, $K_c$, $n_H$, and $n_c$, are the binding parameters. The next three quantities are known from the experimental conditions or measurements. The next five quantities, $\phi_H$, $\phi_C$, $L$, $P_H$, and $P_C$ are intermediate variables and the last two, $v_H$ and $v_C$, are the required binding densities. The necessary equations follow.

$$P_H = DNA(1 - Hyp)$$

(Eq. 4)

$$P_C = DNA - Hyp$$

(Eq. 5)

$$\phi_H = (1 - n_H \cdot v_H)/(1 - n_H \cdot v_H)$$

(Eq. 6)

$$\phi_C = (1 - n_C \cdot v_C)/(1 - n_C \cdot v_C)$$

(Eq. 7)

$$L = L_T/(1 + K_H \cdot P_H \cdot \phi_H + K_C \cdot P_C \cdot \phi_C)$$

(Eq. 8)

$$v_H = L \cdot K_H \cdot \phi_H$$

(Eq. 9)

$$v_C = L \cdot K_C \cdot \phi_C$$

(Eq. 10)

These equations are based on those given by McGhee and von Hippel (1974) and developed by McGhee (1976) and

<table>
<thead>
<tr>
<th>Table IV</th>
<th>Quantities considered in the determination of the binding density</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_H$</td>
<td>Association coefficient for ligand binding to double helical DNA</td>
</tr>
<tr>
<td>$K_c$</td>
<td>Association coefficient for ligand binding to random coil DNA</td>
</tr>
<tr>
<td>$n_H$</td>
<td>Size of binding site on double helical DNA (bases)</td>
</tr>
<tr>
<td>$n_c$</td>
<td>Size of binding site on random coil DNA (bases)</td>
</tr>
<tr>
<td>$L_T$</td>
<td>Total ligand concentration (mol/l)</td>
</tr>
<tr>
<td>Hyp</td>
<td>Fractional hyperchromicity (0-1)</td>
</tr>
<tr>
<td>DNA</td>
<td>Total DNA concentration (mol/l DNA phosphate)</td>
</tr>
<tr>
<td>$\phi_H$</td>
<td>Excluded binding site factor for double helical DNA</td>
</tr>
<tr>
<td>$\phi_C$</td>
<td>Excluded binding site factor for random coil DNA</td>
</tr>
<tr>
<td>$L$</td>
<td>Free ligand concentration (mol/l)</td>
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<tr>
<td>$P_H$</td>
<td>Conc. of polymer in helix conformation</td>
</tr>
<tr>
<td>$P_C$</td>
<td>Conc. of polymer in coil conformation</td>
</tr>
<tr>
<td>$v_H$</td>
<td>Binding density on double helical DNA</td>
</tr>
<tr>
<td>$v_C$</td>
<td>Binding density on random coil DNA</td>
</tr>
</tbody>
</table>
Morgan et al. (1986). They were solved numerically. Values for the first seven quantities in Table IV were provided to a computer worksheet, and it generated values for \( u_H \) and \( v_C \).

We proceeded as follows. 1) Select values (the current estimate) for the binding parameters \( K_H, K_C, n_H, \) and \( n_C \). 2) Calculate the binding density on double-helical DNA, \( u_H \), at the \( T_m \) (\( Hyp = 0.5 \)) for each ligand concentration, using \( u_H = f(K_H, K_C, n_H, n_C, L_T, P_T, P_C) \). 3) Construct the equation \( \Delta T = g(u_H) \) by plotting the measured \( \Delta T \) at 50% hyperchromicity as a function of \( u_H \). A quadratic function was used for interpolation since it gave a good fit in all cases. 4) Calculate \( u_H \) at \( Hyp = 0.2 \) and \( Hyp = 0.8 \) for each ligand concentration. 5) From the \( \Delta T = g(u_H) \) curve, read off the predicted \( \Delta T \) at \( Hyp = 0.2 \) and \( Hyp = 0.8 \). 6) Calculate the difference between the predicted and experimental \( \Delta T \) for each ligand concentration, at the two \( Hyp \) values. Square these differences and add them to give a total square error. 7) Repeat the process with different values of the binding parameters and construct a four-dimensional matrix of total square error values as a function of the four parameters. Use pattern searching to determine the values of the binding parameters that give the best fit (minimum total square error) to the data.

Results and Discussion—The procedure was partly automated and about 200 different combinations of values of the binding parameters were tested. An apparently unique solution was obtained. We have looked hard for other solutions, starting with parameters far removed from the best fit quoted. In all cases, the path of minimizing the error led directly to the solution that was found. Hence, the values obtained are believed to represent a unique solution. A clear minimum total square error was obtained for all four parameters in the case of the non-acetylated peptide ligand. For the acetylated peptide ligand, three parameters gave a clear minimum and the fourth parameter, \( n_C \), gave essentially the same result for all values less than approximately 1. This probably reflects the absence of tightly localized binding, which is consistent with the low association constant.

There may be partial correlations between the binding parameters. For example, in the original work by Morgan et al. (1986) the fit depended much more strongly on the ratio of binding constants than on the constants themselves, although in most cases there was sufficiently strong dependence on the absolute values. In the present study, no correlations between the binding parameters have been noted.

The root mean square error between predicted and experimental melting temperatures was plotted as a function of each binding parameter, for the two ligands, to demonstrate the existence of a minimum error, in Figs. 8 and 9. Only values near the minimum are presented in Figs. 8 and 9, but many other values for the binding parameters were investigated and no other minima were found. The minimum root mean square error for the complete data set was 0.55 °C for the acetylated peptide and 0.45 °C for the non-acetylated peptide. This residual error represents partly experimental uncertainty. There may also be errors due to the analysis ignoring end effects; the DNA was only approximately 10 times longer than the binding site size. However, the substantial agreement, about 0.5 °C, between the predicted and experimental results supports the basic assumptions and indicates the accuracy of the determinations of the binding parameters. In Fig. 10, the experimental thermal denaturation curves are plotted as continuous lines and the predicted curves have been calculated at 0.1 hyperchromicity intervals and plotted as discrete points. Fig. 10 shows graphically the fit between the values.

It is difficult to make a purely objective estimate of the errors in the binding parameters. The four-dimensional matrix of mean square error values as function of the four parameters shows objectively how the parameters affect the mean square error. However, we must subjectively supply a
cut-off value for the root mean square that is acceptable. If the experimental uncertainty in the melting temperature measurements is ±0.5 °C and if there are additional uncertainties in determining $g(r_0)$, we can subjectively rule out parameters that give a root mean square error greater than 0.6 °C. This criterion has been used to estimate errors from the four-dimensional matrix, and the results are given in Table I.

This appears to be the first application of the excluded sites model to large ligand binding sites ($n_u > 4$). The small error obtained provides experimental confirmation of this model.

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