Site-specific Binding Affinities within the H2B Tail Domain Indicate Specific Effects of Lysine Acetylation

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Acetylation of specific lysines within the core histone tail domains plays a critical role in regulating chromatin-based activities. However, the structures and interactions of the tail domains and the molecular mechanisms by which acetylation directly alters chromatin structures are not well understood. To address these issues we developed a chemical method to quantitatively determine binding affinities of specific regions within the individual tail domains in model chromatin complexes. Examinations of specific sites within the H2B tail domain indicate that this tail contains distinct structural elements and binds within nucleosomes with affinities that would reduce the activity of tail-binding proteins 10–50-fold from that deduced from peptide binding studies. Moreover, we find that mutations mimicking lysine acetylation do not cause a global weakening of tail-DNA interactions but rather the results suggest that acetylation leads to a much more subtle and specific alteration in tail interactions than has been assumed. In addition, we provide evidence that acetylation at specific sites in the tail is not additive with several events resulting in similar, localized changes in tail binding.

Nucleosomes are the fundamental repeating subunits of eukaryotic chromatin, comprising about 200 bp of DNA each, 147 bp of which are wrapped about 1.65 times around an octamer of the four core histone proteins (1, 2). Strings of nucleosomes are assembled into secondary structures such as the 30 nm diameter chromatin fiber and the higher order tertiary structures perhaps exemplified by the ~400 nm chromonema fibers (3–6). The organization of nucleosomes within secondary and tertiary chromatin structures and the molecular interactions responsible for their formation are poorly understood (4, 7, 8).

Approximately 75% of the mass of each of the core histones is organized into a largely α-helical domain that is assembled into the protein spool onto which the DNA is wrapped (1, 9). The remaining mass is contained with the core histone tail domains, which project out from the interior of the nucleosome core and are accessible to both DNA and protein targets in chromatin (10–12). The tails are essential for formation of higher order secondary and tertiary chromatin structures and participate in short and long range inter-nucleosome interactions (5, 7, 13, 14). Although the tails adopt random coil conformations when released from their binding sites in moderate ionic strength solutions (>0.4 M NaCl) (15–18) and are often referred to as “unstructured” domains, evidence suggests they adopt defined secondary and tertiary chromatin structures and participate in short and long range inter-nucleosome interactions (5, 13, 14). Although the tails adopt random coil conformations when released from their binding sites in moderate ionic strength solutions (>0.4 M NaCl) (15–18) and are often referred to as “unstructured” domains, evidence suggests they adopt defined structures and participate in local interactions that vary with ionic strength (19). However, in general the structures and interactions of the tail domains are poorly understood.

The core histone tail domains play key roles in the epigenetic regulation of gene expression, and post-translational modifications such as acetylation of specific lysines within these domains are closely linked to transcriptionally active regions of the genome (25–27). Lysine acetylation can function as an epigenetic “mark” facilitating the recruitment of additional factors to specific loci to facilitate transcription (28, 29). In addition, acetylation within the tail domains directly alters the accessibility of nucleosomal DNA and reduces the ability of nucleosome arrays to assemble into higher order structures (5, 30–34). However, the mechanism by which lysine acetylation directly alters the structure and functionality of chromatin remains unclear. Acetylation results in neutralization of the positive charge on lysine, and thus it is often assumed that acetylation weakens histone-DNA interactions, resulting in a more open and transcriptionally permissible chromatin structure (35, 36). However, recent evidence suggests that the effect of acetylation may be much more complex. For example, acetylation of lysine 16 within the H4 tail domain abrogates an interaction between the H4 tail and a surface of H2A/H2B histone fold domains contributing to stability of the folded chromatin fiber (1, 7, 24). However, in general the structures and interactions of the tail domains are poorly understood.

**References**

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core histone tail domains. In this study, we focused on histone H2B tail-DNA interactions and the effect of acetylation on this domain. Our results indicate that acetylation in the H2B tail domain does not cause a global weakening of tail interactions but rather induces specific alterations in tail structure.

**EXPERIMENTAL PROCEDURES**

**Preparation of Core Histones**—Coding sequences for H2B mutants were obtained from the wild type *Xenopus* H2B sequence by the Stratagene QuikChange kit and were cloned into the pET3a expression plasmid. Bacterial cells expressing these proteins were resuspended in 10 mM EDTA, 0.5% Triton X-100 and incubated on ice for 30 min. The mixture was then acidified with HCl to a final 0.4M, incubated on ice for 30 min, X-100 and incubated on ice for 30 min. The mixture was then digested with micrococcal nuclease (70 units/ml) at 37 °C for 10 min in 10 mM Tris, pH 8.0, 2 mM CaCl2 to generate NCPs. The digestion was stopped by adding EDTA to 2.5 mM, and NCPs were reconstituted by salt dialysis in the presence of 10 mM DTT.2 (H3/H4)2 tetramers were purified from the chicken red blood cell nuclei as described (39).

**Reconstitution of Nucleosomes and Purification of Nucleosome Core Particles**—Calf thymus DNA was sonicated into ~1–2-kb fragments. H2A/H2B, H3/H4, and calf thymus DNA were reconstituted by salt dialysis in the presence of 10 mM β-mercaptoethanol and then condensed to 2.5 mg/ml and digested with micrococcal nuclease (70 units/ml) at 37 °C for 10 min in 10 mM Tris, pH 8.0, 2 mM CaCl2 to generate NCPs. The digestion was stopped by adding EDTA to 2.5 mM, and NCPs were purified on 5–20% sucrose gradients centrifuged at 4 °C for 18 h at 34,000 rpm.

**Reconstitution of Nucleosome Arrays and Purification of Mononucleosomes**—The 208-12 DNA template, which contains 12 tandem 208-bp repeats of *Lytechinus* 5 S rDNA, was prepared and reconstituted as described (40). The molar ratio of histone octamer to 5 S DNA repeat DNA was maintained at 0.9 to produce subsaturated nucleosome arrays. 5 S mononucleosome was purified on 5–20% sucrose gradients centrifuged at 4 °C for 18 h at 34,000 rpm.

**Reaction of Nucleosomes and H2A/H2B Dimers with Fluorescein 5-Maleimide**—NCPs containing H2B cysteine mutations were incubated in 10 mM DTT for 1–2 h at room temperature. and then DTT was removed by exhaustive buffer exchange to 10% glycerol TE using an Amicon YM-10 concentrator. NCPs were rapidly frozen and stored at −80 °C for extended periods. Samples of 0.8 µM NCPs containing different NaCl concentrations were reacted with 5.6 µM FM for different times, and the reaction was then stopped by adding DTT to a final concentration of 5 mM. 5 S mononucleosomes and arrays were treated in the same way. Reactions of free H2A/H2B dimers with FM was performed by chemical quench-flow (Kintec) to examine reaction points in the millisecond time scales. Reactions were initiated by mixing equal volumes of dimers (1.6 µM) and FM (11.2 µM) for various times, and then the reaction was quenched with 4 volumes of 10 mM DTT. In selected cases urea was added to 5 M final concentration to the reactions to induce unfolding of the dimers (41).

**Determination of Reaction Rate Constants**—The extent of FM conjugation with H2Bs was analyzed by separation of samples in 15% SDS-polyacrylamide gels, and then either whole gel fluorimetry (GE Healthcare) or digital photography of gels was illuminated by 365 nm light on a UV light box. The images were analyzed using ImageQuant (GE Healthcare) software, and reaction rate constants and global fits were determined using GraphPad Prism 4 software. Band volumes were determined at appropriate time intervals (A), and a maximal extent of reaction (A0) was determined from points in the curve where d(A)/dt = 0. kNCP was taken from fits of the standard single phase exponential equation, A = A0(1 − e−kt). Errors were propagated by the partial differential method from source values. The free H2A/H2B dimer and U react with FM with identical kinetics (see below and Equation 3),

$$k_3 D + FM \rightarrow D-FM$$

(Eq. 3)

The B and U form are related by a conformational equilibrium constant (see Ref. 42) as shown in Equation 4,

$$K_{eq} = \frac{k_{12}}{k_{21}}$$

(Eq. 4)

Thus the reaction rate can be defined as shown in Equation 5,

$$V = k_3[FM][U]$$

(Eq. 5)

Because the concentration of FM we used, [FM], is >5 times higher than that of NCPs, [FM] can taken as a constant, and Equation 5 becomes Equation 6,

$$V = k_3'[U]$$

(Eq. 6)

in which the pseudo reaction rate constant \(k_3'\) is defined as shown in Equation 7,

$$k_3' = k_3[FM]$$

(Eq. 7)

Applying the steady state condition for the intermediate U, Equation 8 is given,

$$\frac{d(U)}{dt} = 0$$

(Eq. 8)

and considering the reactant NCPs, as shown in Equation 9,
NCP = B + U  \hspace{1cm} \text{(Eq. 9)}

and that the reaction of NCPs with FM can be described by overall rate constant $k_{NCP}$ as shown in Equation 10,

$$k_{NCP} = -N^{-1}\text{d}(N)/\text{d}t$$  \hspace{1cm} \text{(Eq. 10)}

we have Equation 11,

$$k_{NCP} = k_{12}k_3/(k_{12} + k_{21} + k_3')$$  \hspace{1cm} \text{(Eq. 11)}

There are two limiting cases for Equation 11 (see Refs. 42, 43).

**Case I**—Slow conformational transition limit is shown in Equation 12,

$$k_3' >> k_{21}$$  \hspace{1cm} \text{(Eq. 12)}

In this limit, Equation 11 is reduced to Equation 13,

$$k_{NCP} = k_{12}k_3/(k_{12} + k_3')$$  \hspace{1cm} \text{(Eq. 13)}

**Case II**—Rapid conformational pre-equilibrium limit, which is opposite to the case I, is shown in Equation 14,

$$k_3' << k_{21}$$  \hspace{1cm} \text{(Eq. 14)}

Now Equation 11 can be rewritten as Equation 15,

$$k_{NCP} = k_3'/K_{eq}/(1 + K_{eq})$$  \hspace{1cm} \text{(Eq. 15)}

At very low salt concentrations, $K_{eq}$ should less than 1. So, for case I, Equation 16,

$$k_3' >> k_{12}$$  \hspace{1cm} \text{(Eq. 16)}

gives Equation 17,

$$k_{NCP} = k_{12}$$  \hspace{1cm} \text{(Eq. 17)}

For case II, combining Equation 7, we find that $k_{NCP}$ is linearly proportional to the [FM] in the reaction (see below). Thus the dependence of $k_{NCP}$ on [FM] can be used to distinguish these two models. For case II, this dependence is first-order, whereas case I is zero order.

Fig. 1E shows that the dependence of the overall reaction rate constant, $k_{NCP}$, on [FM] at two different NaCl concentrations is first order. So case II, rapid conformational pre-equilibrium limit, applies. Thus from Equations 8, 14, and 15 we have Equation 18,

$$U/B = k_{12}/(k_{21} + k_3') = k_{12}/k_{21} = K_{eq}$$  \hspace{1cm} \text{(Eq. 18)}

and by rearranging we have Equation 19,

$$K_{eq} = k_{NCP}/(k_3' - k_{NCP})$$  \hspace{1cm} \text{(Eq. 19)}

As mentioned above and in the text, we assume that $k_3'$, the intrinsic rate of reaction of the NCP U form with FM, is equivalent to the intrinsic rate of reaction for the corresponding free H2A/H2B dimer. Both the U form of NCPs and free dimer have highly mobile tail domains (16, 18). Indeed we find that the rate of reaction of the free dimer and the NCP at elevated salts (0.5 M) where the tails are expected to be completely released from binding sites within nucleosomes (17) are equivalent (see text).

**RESULTS**

Despite numerous studies of core histone tail domains, actual binding affinities of these domains in the context of nucleosomes have not been determined. We reasoned that evaluation of the binding parameters for several sites within a tail domain would reveal fundamental aspects of tail function and of post-translational modifications within these domains. Thus we developed an assay based on a prior observation that the reactivity of cysteines placed within the histone tail domains reflected the binding state of the tail; very low reactivity was observed in low salt (TE) conditions, whereas reactivity was greatly enhanced when the ionic strength was raised to levels expected to shift the equilibrium toward a state where the tails were dissociated from the nucleosome surface.3 We focused on the H2B tail domain for these initial studies, first examining the reactivity of a cysteine substituted at the 6th position in the H2B tail domain (Fig. 1A, H2BS6C) with FM. Note that cysteine is nearly isosteric with serine, and thus no significant alterations of tail interactions are expected from this single substitution. Reaction of NCPs containing H2B S6C with FM resulted in specific modification of H2B (Fig. 1B) and exhibited first-order kinetics, with an apparent rate constant $k_{NCP}$ that increased with increasing concentrations of NaCl in the reaction (Fig. 1C, see “Experimental Procedures”). A plot of $k_{NCP}$ versus [NaCl] (Fig. 1D) shows the overall reaction rate increases up to about 500 mM NaCl, a salt concentration at which the tails are completely dissociated from the nucleosome surface (15, 17).

However, $k_{NCP}$ describes the overall reaction as shown in Reaction 1 (where Nuc indicates nucleosome),

\[
\text{Nuc}_{\text{tail bound}} \rightleftharpoons \text{Nuc}_{\text{tail unbound}} + \text{FM} \rightarrow \text{Nuc-FM}
\]  \hspace{1cm} \text{REACTION 1}

Examination of the dependence of $k_{NCP}$ on [FM] indicates that this reaction behaves according to a pre-equilibrium kinetic model (Fig. 1E and see “Experimental Procedures”). Thus, the tail-bound and tail-unbound states are in equilibrium, and it is possible to determine a conformational equilibrium constant, $K_{eq}$, relating the concentrations of these species as shown Equation 20,

$$K_{eq} = \frac{\text{Nuc}_{\text{tail unbound}}}{\text{Nuc}_{\text{tail bound}}}$$  \hspace{1cm} \text{(Eq. 20)}

However, calculation of $K_{eq}$ requires determination of $k_3'$, reflecting the intrinsic rate of reaction of the unbound tail with FM. We assumed that the intrinsic rate of reaction of FM with the tail-unbound (nucleosome) species is equivalent to the rate of the reaction of free H2A/H2B S6C dimers with FM. In support of this assumption, we note that the rates of FM reaction with NCPs containing H2B S6C at ≥500 mM NaCl, where the tails are expected to be completely dissociated from the nucleosome surface, are equivalent to the rates observed with free H2A/H2B S6C dimers at the same salt concentration (results

3 K.-M. Lee and J. J. Hayes, unpublished observations.
A plot of $k_3'$ (see “Experimental Procedures”) versus [NaCl] as determined by reaction of the free H2A/H2B S6C dimers with FM shows that the intrinsic rate of the reaction is salt-dependent in a manner opposite to the salt dependence observed for $k_{NCP}$ (Fig. 1F). Repeating the assay at selected [NaCl] in the presence of 5mM urea indicates that the reaction rates for the free protein are not influenced by salt-dependent alterations in protein structure (Fig. 1F, diamonds).

Calculation of $K_{eq}^{S6C}$ over a range of [NaCl] (Fig. 1, G and H) yields data that can be fit by a sigmoidal curve and describes the release of the tail from the nucleosome surface, with the steepest dependence of $K_{eq}^{S6C}$ on [NaCl] between 150 and 500mM NaCl. $K_{eq}^{S6C}$ values increase more than 2000-fold from 0 to 500mM NaCl (0.00152 at 0mM and 3 at 500mM NaCl). The data indicate that the region of the H2B N-terminal tail encompassing residue 6 binds very tightly within NCPs at very low ionic strengths. However, at physiological salt (150mM NaCl) $K_{eq}^{S6C}$ is about 0.1 indicating that the end of the H2B tail is quite mobile, spending about 1/10 of the time dissociated from the nucleosome surface. Importantly, the data are consistent with previous quantitative determinations of the effect of salt on tail binding behavior (15, 18, 44–46), in support of the contention that we have determined $K_{eq}^{S6C}$ over the range of [NaCl] for the end of the H2B tail domain. We also note that the nucleosomes used in this analysis remained intact over the range 0–500mM NaCl as expected (Fig. 2 and results not shown). As also indicated by the lack of labeling of the cysteine within H3 at position 110 (Fig. 1B).

**FIGURE 1. Determination of equilibrium binding constants for the H2B tail domain.** A, schematic of the H2B N-terminal tail domain. Sites of cysteine substitutions used in this study are indicated. B, representative tail modification time course. NCPs were incubated in 150mM NaCl then reacted with FM for the indicated times. Samples were analyzed by SDS-PAGE followed by fluorography and Coomassie staining. C, determination of $k_{NCP}$. Reactions such as shown in B at various [NaCl] as indicated were quantified as described under “Experimental Procedures” and data fit to a first order rate equation with slope $-k_{NCP}$. In all cases $r^2 > 0.95$. D, plot of $k_{NCP}$ versus [NaCl] for H2B S6C in NCPs. Data from fits as shown in C were plotted versus NaCl concentrations. Error bars represent two standard deviations derived from at least three determinations of $k_{NCP}$ at each [NaCl]. Data were fitted to a Boltzmann sigmoidal curve ($r^2 = 0.9931$). E, dependence of $k_{NCP}$ on [FM] at 0 and 150mM [NaCl] (left and right axes, respectively) indicates that the reaction behaves according to a pre-equilibrium kinetic model (see “Experimental Procedures”). F, plot of $k_3'$, the intrinsic rate of H2B S6C reaction with FM versus [NaCl]. Data were obtained by reaction of free H2A/H2B S6C dimers with FM. The reaction at selected [NaCl] was repeated in the presence of 5mM urea as indicated by the filled diamonds. Data were fit to a one-phase exponential decay curve. G and H, independent $K_{eq}$ values at various [NaCl] were calculated from data as shown in D and F, then plotted. Note that at elevated [NaCl], $K_{eq}^{S6C}$ approaches $k_3'$ and $K_{eq}^{S6C} > 1$ becomes indeterminate, thus only the lower range [NaCl] data are plotted. G, $K_{eq}$ for NCPs containing H2B S6C, H2B S14C, or H2B T21C versus [NaCl]; H, same data shown in G, expanded over the range 0–200mM NaCl. Data were fitted to Boltzmann sigmoidal curves as in D.

**Other Sites in the H2B Tail**—We next determined $K_{eq}$ values for two other sites within the ~27-amino acid H2B tail domain by substitution of residues at positions 14 or 21 for cysteine (Fig. 1A). Interestingly, we find that these sites exhibit about
a 10-fold greater binding affinity at low salt concentrations and a 5-fold tighter association within the nucleosome in the range of physiological ionic strengths (150 mM NaCl) compared with site 6 (Fig. 1H). The dependence of the conformational equilibrium constants on ionic strength at sites 14 and 21 also fit sigmoidal curves as observed at site 6, with $K_{eq}$ values at these two sites increasing more than 10,000-fold from 0 to $\sim$500 mM NaCl ($\sim$0.0001 at 0 mM and $\sim$1 at 500 mM NaCl). Moreover, the affinities of both sites are identical from 0 to $\sim$350 mM [NaCl] suggesting that both sites are components of the same structural element in the H2B tail when bound to DNA, and distinct from site 6.

Effect of Acetylation Mimics on Tail Binding Affinity—We next attempted to gain insight to the direct effect of acetylation on tail interactions by examining the binding behavior of H2B N-terminal tail domains containing Lys $\rightarrow$ Gln substitutions to mimic lysine acetylation. Glutamine has a neutral side chain that resembles acetylated lysine in charge and structure and can functionally replace this modification $\rightarrow$Gln mutations at sites 15, 24, and 27 had the same $K_{eq}$ values at physiological salt concentrations (150 mM NaCl) as that observed with the 4 and 6 Lys $\rightarrow$ Gln mutants (Fig. 4C), whereas substitutions sites 12 and 20 resulted in alterations in $K_{eq}$ for the native (unacetylated) H2B S14C tail attains a maximal value at $\sim$50 mM NaCl and an $\sim$2–3-fold increase in the range of 150 mM NaCl (Fig. 4, A and B). Moreover, we find that multiple Lys $\rightarrow$ Gln mutations caused increases in $K_{NCP}$ similar or equivalent to that caused by the single changes. For example, single Lys $\rightarrow$ Gln mutations at sites 15, 24, and 27 had the same $K_{eq}$ values at physiological salt concentrations (150 mM NaCl) as that observed with the 4 and 6 Lys $\rightarrow$ Gln mutants (Fig. 4C), whereas substitutions sites 12 and 20 resulted in alterations in $K_{eq}$ for the native (unacetylated) H2B S14C tail attains a maximal value at $\sim$50 mM NaCl, while a maximal value for the multiply "acetylated" tail is attained at $\sim$150 mM NaCl (Fig. 4E). Similar results were obtained when comparing double mutants at sites 12 and 15 with the multiple site mutants (Fig. 4D) suggesting the effects of acetylation in the region of residue 14 within histone H2B tail domain are not additive. In general mutations modeling acetylation cause about a 10-fold decrease in binding affinity in $K_{eq}$ at low salt concentrations, about 3-fold at moderate salt conditions (150 mM), and decreasing differences at higher [NaCl] as the tails become fully dissociated from the nucleosome surface.

The effects of mutations modeling acetylation at residue 21 (H2B T21C6KQ), a position near the histone fold domain, appear to be intermediate with respect to that measured at binding affinities (Fig. 3, A and B). Substitution of Lys-5, Lys-12, Lys-20, or Lys-27 for Gln resulted in no significant change in the range of 0 to $\sim$200 mM NaCl, whereas substitution of Lys-15 and Lys-24 resulted in very modest increases of about a 1.2-fold in $K_{eq}$ at 150 mM NaCl with diminishing differences at lower NaCl concentrations.

Because multiple acetylation events often occur together on the same tail, we next examined multiple Lys $\rightarrow$ Gln substitutions in the H2B tail domain. Surprisingly, combinations of 4 or 6 Lys $\rightarrow$ Gln substitutions within the same tail (H2B S6CK5Q,K12Q,K15Q,K20Q (H2B S6C4KQ) and H2B S6CK5Q,K12Q,K15Q,K20Q,K24Q,K27Q (H2B S6C6KQ)) in NCPs did not result in further significant changes in the conformational equilibrium constant $K_{eq}$ at any [NaCl] examined (Fig. 3, C and D). Because $k_{eq}$, reflecting the intrinsic rate of cysteine reactivity, was nearly identical for all 6G C glutamine substitution mutants tested (supplemental Fig. S1), we compared $K_{NCP}$ data for each of these mutants directly (Fig. 3, E and F). This analysis allowed comparison over a more extensive range of [NaCl] than is possible via calculation of $K_{eq}$ (see “Experimental Procedures”) and shows that $K_{NCP}$ values for the wild type H2B tail and Lys $\rightarrow$ Gln mutant proteins are virtually identical over the range 0–1 M NaCl. These data indicate that mutations modeling acetylation do not cause a wholesale weakening of the binding affinity for the region of the H2B tail domain encompassing residue 6.

We next examined the effect of mutations modeling acetylation events at site 14 within the H2B tail domain in NCPs. In contrast to the effect on $K_{eq}$, most single Lys $\rightarrow$ Gln mutations resulted in significant increases in $K_{eq}$ at position 14. For example, substitution of lysines at all positions except residue 5 with glutamine resulted in an $\sim$10-fold increase in $K_{eq}$ at $\sim$50 mM NaCl and an $\sim$2–3-fold increase in the range of 150 mM NaCl (Fig. 4, A and B). Moreover, we find that multiple Lys $\rightarrow$ Gln mutations caused increases in $K_{eq}$ similar or equivalent to that caused by the single changes. For example, single Lys $\rightarrow$ Gln mutations at sites 15, 24, and 27 had the same $K_{eq}$ values at physiological salt concentrations (150 mM NaCl) as that observed with the 4 and 6 Lys $\rightarrow$ Gln mutants (Fig. 4C), whereas substitutions sites 12 and 20 resulted in alterations in $K_{eq}$ that were not statistically different from the tails containing multiple mutations in this range. Interestingly, $K_{NCP}$ for the native (unacetylated) H2B S14C tail attains a maximal value at $\sim$50 mM NaCl, while a maximal value for the multiply "acetylated" tail is attained at $\sim$150 mM NaCl (Fig. 4E). Similar results were obtained when comparing double mutants at sites 12 and 15 with the multiple site mutants (Fig. 4D) suggesting the effects of acetylation in the region of residue 14 within histone H2B tail domain are not additive. In general mutations modeling acetylation cause about a 10-fold decrease in binding affinity in $K_{eq}$ at low salt concentrations, about 3-fold at moderate salt conditions (150 mM), and decreasing differences at higher [NaCl] as the tails become fully dissociated from the nucleosome surface.

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![FIGURE 2. Reconstituted NCPs are stable in 0–500 mM NaCl. Purified NCPs were adjusted to different NaCl concentrations and sedimented through 5–20% sucrose gradients containing the same salt concentrations. Fractions were analyzed by electrophoresis on 0.7% nucleoprotein gels (left) and 15% SDS-polyacrylamide gels (right). Lane M contains 1 kb plus DNA size marker, and lane N shows the purified NCPs before salt treatment and gradient fractionation. Note the expected salt-dependent alteration in sedimentation coefficient evident in the 250 and 500 mM experiments (2) and the nucleosome dissociation into H2A/H2B dimers and H3/H4 tetramer-DNA complexes in gradients containing 1 M NaCl.](image-url)
Effect of Acetylation on H2B Tail Interactions

positions 6 and 14. An H2B tail with 6 Lys → Gln mutations result in only a modest increase in $K_{eq}$, about 1.5-fold, at physiological salt ionic strengths (Fig. 5, A and B). Thus acetylation appears to have much greater effects on binding of the middle of the H2B tail domain than the N- or C-ends of this domain.

The native H2B tail contains 9 lysines and no other positively charged residues. Surprisingly, as described above there was no alteration in tail affinity as reflected by $K_{eq}^{S6C}$ when 6 of the 9 lysines were substituted for glutamines. Moreover, although 6 Lys → Gln substitutions induced significant changes in cysteine reactivities at positions 14 and 21, our analysis indicates that the H2B tail in these regions still exhibits salt-dependent binding behavior with $K_{eq}$ values approaching $2 \times 10^{-4}$ at low salt (TE) conditions. Because it is assumed that the vast majority of binding free energy is contributed by electrostatic interactions of positively charged residues with the polyanionic backbone of DNA, we investigated this issue further by mutating all 9 lysines to glutamine and measuring the tail binding strength in the NCPs. Surprisingly, we find that the tail still exhibits similar not induce significant condensation of H1-lacking nucleosome arrays (5, 55). We found that $K_{eq}$ values for the 6th position within the H2B tail were not significantly different when measured within mononucleosomes and arrays containing H2B S6C from those found for NCPs (Fig. 7, A and B). Similar results were obtained for nucleosome arrays containing H2B S14C or H2B T21C (Fig. 7, C and D). However, we did detect a small but significant decrease in the binding affinity at position 14 for NaCl concentrations ≥200 mM in the nucleosome array that may be caused by the relocation of the histone H2B tail domain from the nucleosome to linker DNA (52). These data also indicate that our results are not dependent upon specific DNA sequences as NCPs that contain essentially random DNA sequences exhibited affinities identical to that measured with nucleosomes assembled with 5S DNA sequences (Fig. 7A).

DISCUSSION

We have developed a quantitative method to measure conformational equilibrium constants reflecting the affinity with which core histone tail domains and regions within these binding affinities at all three sites probed in the 9 Lys → Gln mutants as were found in the 6 Lys → Gln mutants (Fig. 6 and results not shown). Thus, tails completely lacking positively charged residues and containing only a single positive charge at the N terminus of the protein still exhibited a distinct salt-dependent binding behavior. These data suggest that constituents other than the positively charged lysine residues make significant ionic strength-dependent contributions to the binding free energy of the H2B tail domain (see below).

**Linker DNA and Specific DNA Sequences Have No Effect on H2B Tail Binding Affinity**—The addition of linker DNA to NCPs can lead to a relocation of histone tail-DNA interactions and increase histone tail-DNA cross-linking (22, 52, 53). To determine whether the presence of linker DNA has any effect on the observed binding affinity of the H2B tail domain, we reconstituted nucleosome arrays using the *Lytellinus variegates* 208-12mer array DNA template (54) and our recombinant core histones, including H2B cysteine substitution mutants. Arrays were reconstituted at a substoichiometric ratio of histone octamer to 208-bp repeat to eliminate any complication from NaCl-dependent folding of the array (5). Note also that monovalent salts do not induce significant condensation of H1-lacking nucleosome arrays (5, 55). We found that $K_{eq}$ values for the 6th position within the H2B tail were not significantly different when measured within mononucleosomes and arrays containing H2B S6C from those found for NCPs (Fig. 7, A and B). Similar results were obtained for nucleosome arrays containing H2B S14C or H2B T21C (Fig. 7, C and D). However, we did detect a small but significant decrease in the binding affinity at position 14 for NaCl concentrations ≥200 mM in the nucleosome array that may be caused by the relocation of the histone H2B tail domain from the nucleosome to linker DNA (52). These data also indicate that our results are not dependent upon specific DNA sequences as NCPs that contain essentially random DNA sequences exhibited affinities identical to that measured with nucleosomes assembled with 5S DNA sequences (Fig. 7A).
domains bind within any model chromatin complex. This method can be applied over a range of conditions, including a wide range of ionic strengths. Using our method, we find a salt-dependent transition in H2B tail binding affinity that parallels previous general examinations of salt-dependent release of the core histone tails from the surface of nucleosomes (15–17). Indeed, plots of $K_{eq}$ for the H2B tail domain generated by our method almost exactly parallel plots of bulk tail release obtained by NMR of stripped native chromatin (17). The data are also consistent with a more recent examination of salt-dependent tail release by small angle x-ray scattering, which showed a significant increase in the form factor for nucleosome core particles, attributed to tail release, as NaCl concentrations were increased from 10 to 50 mM (45), although no further release was detected in the 50–200 mM range, perhaps because of compensating salt-dependent alterations in core structure. Salt concentrations above 200 mM were not examined in this study. Thus our method can be used to quantitatively determine the binding state of histone tail domains in chromatin complexes.

Moreover, our results bear on the long-standing question of whether the core histone tail domains adopt defined secondary structures when bound within chromatin. The dependence of $K_{eq}$ on ionic strength we detected (Fig. 1H) suggests that sites 14 and 21 are part of the same localized region of cooperative structure, separate and distinct from the region including site 6. Indeed residues 10–21 in the H2B tail have been predicted to form an $\alpha$-helix (56), as supported by spectroscopic evidence (19, 20). Moreover, data from transglutaminase reactivity and fluorescence anisotropy assays indicate two ionic strength-dependent transitions occur within the H2B tail domain, consistent with a cooperative release of the region of the tail encompassing residue 22 (46). Thus, despite the general view of the tails as unstructured domains, these data provide further support for the idea that the tails adopt defined structures and participate in localized interactions when bound within chromatin (38, 57).

![FIGURE 4. Glutamine substitutions mimicking lysine acetylation alter binding of the region encompassing residue 14 within H2B tail domain in NCPs.](image)

![FIGURE 5. Glutamine substitutions mimicking lysine acetylation have only a small effect on binding of the region encompassing residue 21 within H2B tail domain in NCPs.](image)
Although lysine acetylation is intimately linked to actively transcribed chromatin and is known to cause direct alterations in the physical behavior of the chromatin fiber (5), there is little understanding of the actual molecular mechanism(s) by which this modification alters tail-DNA interactions. Although it is often assumed that the basis of this effect is a general weakening of histone tail-DNA interactions, we found that mutations modeling single or multiple acetylation events do not result in a wholesale weakening or loss of tail binding affinity, consistent with earlier UV cross-linking experiments (37). Rather, our data suggest that acetylation causes distinct, localized changes in H2B tail interactions. For example Lys → Gln mutations had no effect or only modest effects on $K_{eq}$S6C and $K_{eq}$T21C, representing interactions of both ends of the H2B tail, but did result in a significant weakening of interactions in the middle of the tail region, indicated by increases in $K_{eq}$S14C. Indeed our results suggest that at physiological ionic strengths acetylation operates like a “switch” such that in the unacetylated tail the region encompassing site 14 exhibits binding similar to site 21, whereas site 14 behaves like site 6 upon acetylation (Fig. 8). This switch may be related to acetylation-dependent alteration of the α-helical content of the tail domains, detected by spectroscopic investigations (19). Nevertheless, our data suggest that the direct effect of acetylation on chromatin structure is derived from a much more subtle and specific alteration in tail interaction than has been previously assumed.

We find that the reaction of FM with cysteine residues within the H2B tail domain can be described by a pre-equilibrium kinetic model. Thus, $k_{31}$ is $> k_{31}'$, indicating that in regions encompassing sites 14 and 21 and in the range of physiological ionic strength $k_{31}$ is more than $\sim 1.7 \text{ s}^{-1}$ and, given $K_{eq}(\sim 0.03)$, then $k_{12}$ is more than $\sim 0.05 \text{ s}^{-1}$, corresponding to half-lives for the bound and unbound states of less than $\sim 15$ and $\sim 0.4$ s. Thus the tail equilibrates fairly rapidly in the context of the nucleosome, indicating there would be little or no kinetic restriction for factors designed to bind to specific sites within the tail domain. Importantly, however, our results imply that binding of such factors would be reduced by 10–50-fold compared with binding affinities measured with free peptides representing the tail domains (58). Of course, such factors may also

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**FIGURE 6.** H2B tails lacking all positively charged residues exhibit salt-dependent binding within NCPs. A and B, comparison of $K_{eq}$ versus [NaCl] for regions encompassing sites 6 and 14 within the H2B tail domain, respectively, for tails containing 0, 6, or 9 Lys → Gln substitutions, as indicated.

**FIGURE 7.** Effect of linker DNA and nucleosome arrays on the H2B tail binding affinities. A, comparison of H2B S6C in NCPs and 5 S mononucleosomes. B–D, comparison of H2B S6C, H2B S14C, and H2B T21C, respectively, in NCPs or nucleosome arrays.

**FIGURE 8.** Site 14 in H2B (S14C) behaves similarly to the region encompassing site 21 before introduction of acetylation mimics and similarly to site 6 after introduction of acetylation mimics.
make productive interactions with sites elsewhere in the nucleosome, increasing affinities (59).

Our work is in agreement with previous work by Dimitrov and co-workers (37) in which UV laser cross-linking revealed no difference in the extent of histone tail-DNA interactions in NCPs containing hypo- and hyper-acetylated nucleosomes. Indeed, we did not find a significant change in tail binding affinity when monitoring reactivity of a cysteine placed at residues 6 or 21, near either end of the tail domain. Nonetheless, we did detect an ∼3-fold increase in $K_{eq}$ at site 14 upon Lys → Gln substitution, indicating a loss of tail binding affinity in this central region. It is important to note, however, that the UV laser cross-linking of Dimitrov and co-workers (37) detected binding of bulk tails, whereas our experiment measures actual changes in affinities. Thus, although we detected a significant drop in affinity for the center of the tail domain upon introduction of mutations modeling acetylation, the actual fraction of tail domains in this region existing in the bound state would have only decreased from ∼98 to ∼93% in the range of 150 mM NaCl, with very little change in flanking regions. Assuming the effect of acetylation is similar in other tail domains, it is not surprising that UV laser cross-linking or other similar techniques sensitive to only the bound state would not detect an alteration in the fraction of tails bound to DNA because of acetylation.

Our data also indicate that acetylation events may not be additive in terms of structural changes or alterations in tail binding induced by this modification. We observed that single Lys → Gln substitutions at lysines 15, 24, and 27 induced as much reduction in tail binding interactions as did multiple substitutions within the H2B tail domain. These results may provide a structural explanation for recent genetic results suggesting that three of four specific lysine acetylation events within the H4 tail domain appear not to be unique in terms of global effect on gene expression in yeast cells (60).

The salt dependence of histone tail binding observed here and in previous reports (15–18) indicates that a portion of the binding free energy of the H2B tail domain is provided by electrostatic interactions, presumably between basic residues and DNA. However, somewhat unexpectedly, our data indicate other significant contributions. For example we find that loss of 6 of the 9 positively charged lysines in the H2B tail did not lead to a complete abrogation of tail binding. Moreover, mutation of all 9 lysines to glutamines did not result in further reductions in affinity at any of the sites probed (Fig. 6). Thus an H2B tail domain devoid of positively charged amino acid residues except for the protonated N-terminal amino group still exhibited tight binding to sites within the NCPs. Moreover, there was remarkably little difference in tail binding affinity at all sites probed between tails with one or two Lys → Gln substitutions and nine Lys → Gln substitutions. Previous work with a free H4 tail domain showed that acetylation vastly reduced binding of an H4 tail peptide to DNA (15, 36). Thus our results indicate that the tail binding in the context of chromatin is significantly different from the binding of free peptides and that constituent lysines other than lysine ε-amino groups participate in salt-sensitive interactions that contribute significantly to tail binding free energy. In addition, it is possible that tail-tail interactions compensate for loss of lysine charge and also that glutamine amides (and secondary amide groups in acetylated lysines) participate in productive (H-bonding) interactions within NCPs as is typically observed in proteins. Indeed lysine acetylation and glutamine substitutions result in similar changes to $K_{eq}$ measured in the H4 tail domain.4 Our results also suggest that unmodified lysine residues may not contribute as significantly to overall tail binding free energy as has been assumed, perhaps because of a severe entropic cost of restricting the conformationally flexible four-carbon side chain and ε-amino group to the vicinity of a negatively charged phosphate. In light of these questions it will be interesting in future experiments to examine the effect of lysine to arginine substitutions within the H2B tail domain because the latter has one less methylene unit and a more conformationally constrained charged moiety and thus might be expected to contribute more positively to the total binding free energy of the tail domain than lysine.

In summary we have for the first time measured conformational equilibrium constants describing the binding affinity of a core histone tail domain. Measurements at specific sites within the tail domain over a range of salt concentrations indicate that the tail contains distinct structural domains and binds with sufficiently high affinities so as to cause significant reductions in the biological activity of tail-binding proteins deduced from peptide binding studies. Moreover, our results suggest that acetylation does not induce a general weakening of tail-DNA interactions as is often assumed but rather causes localized alterations in tail binding. These alterations may be related to specific structural changes in tail structure detected by spectroscopic techniques (19, 57). We present evidence that site-specific acetylation events in the H2B tail domain are not additive and not unique with regard to induced structural changes; many single events induce the same change with regard to binding of the middle of the tail, whereas additional Lys → Gln changes do not induce a commensurate alteration in observed binding affinity. Our results with nucleosomes containing linker DNA and nucleosome arrays suggest that the H2B tail binds with equivalent affinity regardless of the availability of linker DNA (52). It will be interesting to examine whether acetylation or availability of linker DNA causes similar or distinct changes in tail interactions of the other tail domains within the nucleosome or whether acetylation within one tail affects binding of other tails.

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