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Effects of Lysine Acetylation in a β-Hairpin Peptide: Comparison of an Amide$-\pi$ and a Cation$-\pi$ Interaction

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Abstract: The acetylation of lysine is a common posttranslational modification of histone proteins, and the interaction of acetylated lysines with aromatic rings is commonly observed in transcriptionally relevant protein−protein interactions. To determine the nature of this interaction and its potential role in protein structure and function, the effect of lysine acetylation on its interaction with tryptophan has been investigated within the context of a β-hairpin peptide. Acetylation of Lys results in the replacement of a cation$-\pi$ interaction with an amide$-\pi$ interaction. Despite the loss of positive charge, the interaction energy is not significantly perturbed, although the geometry of interaction is influenced such that the amide NH interacts directly with the Trp ring. Thermodynamic analysis indicates an enthalpic driving force for the stabilization, indicating a polar-π interaction. Acyl lysine analogues formyl lysine and trifluoroacetyl lysine were used to further investigate the sterics and electronics of the interaction.

Introduction

The acetylation of lysine is a common posttranslational modification of histone proteins. Lysine acetylation weakens the histone−DNA complex in chromatin by neutralizing the electrostatic interaction between unmodified lysine in histones and the negatively charged phosphate backbone of DNA, thereby activating transcription.[1]Additionally, lysine acetylation is commonly found in cells with DNA damage, where it is also thought to weaken the DNA−histone complex to make DNA accessible to repair enzymes,[2] and can play a role in protein stability by preventing lysine ubiquitination, which tags proteins for proteolysis.[3] The bromodomain, a protein domain with a specific affinity for acetylated lysine, is found in most acyltransferases and in many proteins associated with the regulation of chromatin structure.[4] The bromodomain binding pocket contains highly conserved aromatic residues that appear to interact with acetylated lysine through amide$-\pi$ interactions (Figure 1).[5]

The importance of amide$-\pi$ interactions in structural biology has been long established through data-mining studies of protein crystal structures, in which amide-containing side chains (Gln, Asn) were found to have statistical preference for packing near the faces of aromatic rings.[6] Additional investigations have identified numerous backbone amide$-\pi$ interactions in protein and peptide structures,[6d,7] as well as a number of amide$-\pi$ interactions that occur in the binding of ligands.[8] For example, backbone amide$-\pi$ interactions have been identified between Gly/Phe and Gly/Trp pairs in analyses of pairwise residue

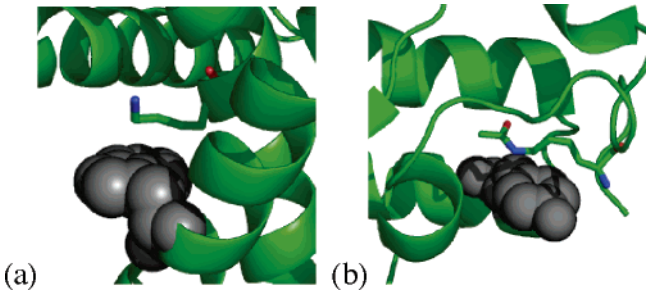

Figure 1. (a) Lys-Trp pair in typical cation$-\pi$ geometry (PDB code: 1ONR). (b) KAc-Tyr pair in typical amide$-\pi$ geometry (Bromodomain from GCN5 complexed with acetylated H4 peptide; PDB code: 1E6I).

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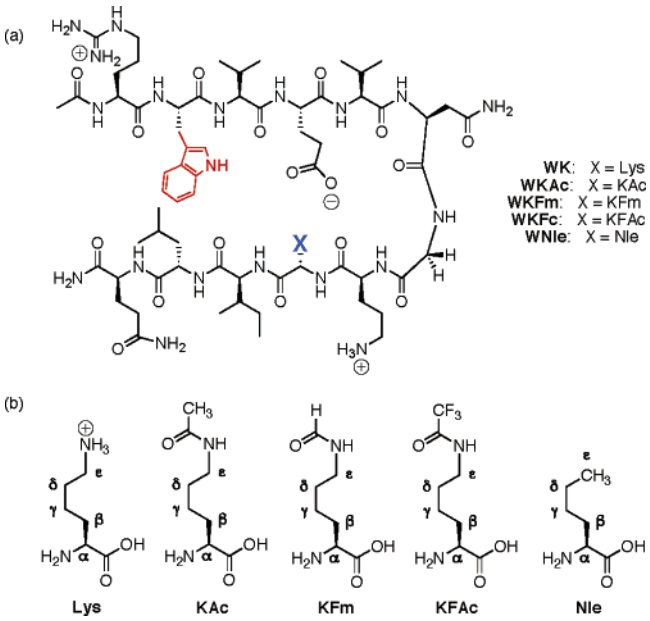

Figure 2. (a) β-Hairpin peptide structure. (b) Amino acids at position X: lysine (Lys), acetyl lysine (KAc), formyl lysine (KFm), trifluoroacetylated lysine (KFAc), and norleucine (Nle).

preferences in antiparallel β-sheets,[9] and amide$-\pi$ interactions have been identified in the binding of the drug bezafibrate by human deoxyhemoglobin.[10] Amide$-\pi$ interactions have also been utilized in the selective binding of guests by model systems in organic solution.[11] Additional theoretical studies have been conducted to demonstrate both the orientation and magnitude of the amide$-\pi$ interaction, using variously the formamide$-$benzene interaction[12] and the ammonia$-$benzene interaction[13] in the gas phase as models for the amide$-\pi$ interaction in proteins. Notably, a recent study investigated both amide$-$ and cation$-\pi$ interactions using implicit solvent models and predicted the amide$-\pi$ interaction of Asn and Gln side chains with an adenine ring (Ade) to be intermediate in magnitude ($-$4 kcal/mol) between the cation$-\pi$ interactions Arg-Ade ($-$7 kcal/mol) and Lys-Ade ($-$2 kcal/mol).[14] In addition, Burley and Pestko's survey of 33 protein crystal structures found higher fractions of polar amide residues interacting with aromatic side chains (31% Asn, 40% Gln) than for Lys (26%), but lower than that of Arg (47%).[6a] These findings suggest that the amide$-\pi$ interaction may be more favorable than the cation$-\pi$ interaction between lysine and an aromatic ring (Figure 1).

To explore the efficacy of amide$-\pi$ interactions, and in particular their role in the recognition of KAc, we have investigated the effects of acetylation of lysine and its interaction with tryptophan within the context of a β-hairpin peptide (Figure 2). This is a useful model system to study noncovalent interactions in water within a biologically relevant context and has previously been used to study cation$-\pi$ interactions.[15] Two analogues of acetyl lysine (KAc), formyl lysine (KFm) and trifluoroacetyl lysine (KFAc), were studied to explore the effects of changes in hydrophobic and electronic character of the acyl group. In our investigation, we find that the interaction between acetylated lysine and tryptophan consists of a polar$-\pi$ interaction that occurs primarily between the polarized amide of KAc and the electron rich face of the Trp indole ring. Despite the loss of the positive charge upon N-acetylation, the KAc$\cdots$Trp interaction is as strong as the unmodified Lys$\cdots$Trp interaction previously reported in the same model system.[15a,b] This indicates that the polar$-\pi$ interaction is energetically competitive with the cation$-\pi$ interaction. This is likely due to a lower desolvation penalty for the interaction of acetyl lysine and tryptophan, while maintaining the electrostatic and van der Waals components of the interaction via NH($\delta+$)$-\pi(\delta-$) and $\pi-\pi$ stacking.

Results and Discussion

Design. The 12-residue β-hairpin sequence used in this study is based on a sequence that has been previously described (Figure 2).[15] Key design features include a good turn-nucleating sequence (Val-Asn-Gly-Orn) and a number of hydrophobic interactions to stabilize the hairpin, including Val3, Val5, Ile10 on one face of the hairpin, and Trp2 and Leu11 on the opposite face of the hairpin. The diagonal interaction between Trp2 and position 9 (residue X in Figure 2) is the site of interest for this study. A Lys residue at position 9 has been previously been shown to interact favorably with Trp2 via a cation$-\pi$ interaction between the ϵ-CH_2 of Lys and the face of the aromatic ring. To explore the relative favorability of an amide$-\pi$ interaction, we investigated the effect of Lys acetylation.

Characterization. The β-hairpin structure and stability was characterized by a number of standard NMR techniques, including α-hydrogen (Hα) chemical shifts, glycine splitting ($\Delta\delta$ Gly), and cross-strand NOEs.[15,16] The degree of Hα downfield shifting and Gly splitting relative to random coil values is used as an indicator of the degree of β-sheet structure at each position along the strand and in the turn, respectively.[17] The fraction folded is calculated with the following equation: $\%_{folded} = (\delta_{obs} - \delta_U)/(\delta_F - \delta_U)$, where the unfolded state (U) is represented by random coil control peptides and the fully folded state (F) by cyclic peptides (see Supporting Information). Furthermore, numerous NOEs between cross-strand pairs of side chains were observed for all peptides, consistent with β-hairpin formation (see Supporting Information).

Effects of Lysine Acetylation. Acetylation of lysine results in a modest enhancement in stability of the β-hairpin peptide. This is demonstrated by an increase in the Hα shifts relative to random coil, as shown in Figure 3a. Based on the glycine splitting, fraction folded of **WKAc** is 87% ($\pm$1%) (side chain $H\alpha_{avg} = 83 \pm 8\%$),[18] versus 78% ($\pm$1%) for the peptide **WK** at 298 K (side chain $H\alpha_{avg} = 76 \pm 7\%$),[18] indicating that

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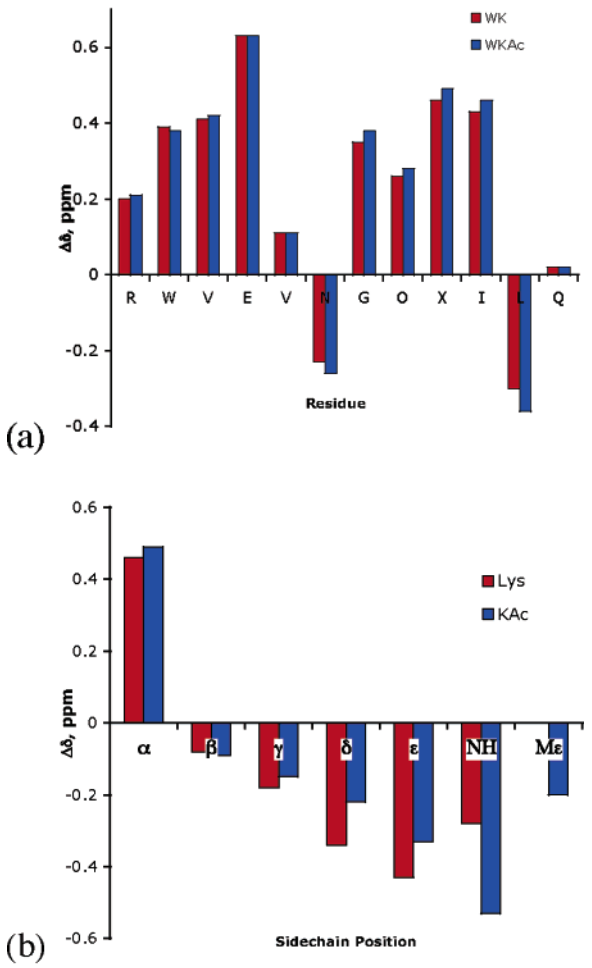

Figure 3. (a) WK and WKAc αH shifts relative to random coil values. Glycine shifts reflect the splitting. (b) WK and WKAc side chain upfield proton shifts relative to random coil values. Conditions: 298K, 50 mM NaOAc-d_3 in D_2O, pH 4.0 (uncorrected), referenced to DSS.

acetylation enhances hairpin stability ($\Delta\Delta G$ = 0.38 kcal/mol, ± 0.05 kcal/mol). Because acetylation of Lys may change its β-sheet propensity, we performed a double mutant cycle to quantitatively assess the interaction energy between Trp and KAc. Val and Ser were used as controls for Trp and Lys, respectively.[15] Double mutant cycles give the magnitude of the Trp···X side chain−side chain interaction for **WK** and **WKAc** to be −0.30 and −0.34 kcal/ mol (±0.1 kcal/mol), respectively. While the differences in the two interaction energies are within experimental error, this is significant in that acetylation does not *decrease* the magnitude of the interaction with Trp, despite the loss of the positive charge. Rather, the conversion of the ammonium group to an amide provides an interaction with Trp of comparable magnitude.

Although the interaction energies for Trp···KAc and Trp···Lys are similar, the upfield shifting patterns of the Lys and KAc side chains indicate a *change in the interaction geometry* with Trp for KAc relative to Lys (Figure 3b). While the unmodified lysine is most upfield shifted at the ϵ-CH_2 protons, KAc is most upfield shifted at its amide proton. The ϵ-CH_2 of KAc exhibits some upfield shifting as well, whereas the acetyl methyl group is less shifted. Thus, although it is difficult to determine if the amide group is stacked with the Trp or interacting via an NH···π interaction, it is clear that it interacts in a different geometry than Lys (Figure 4). The preference for interaction at the amide

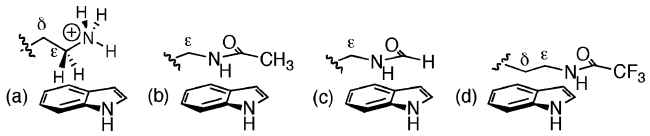

Figure 4. Proposed geometries of interaction for (a) Trp···Lys, (b) Trp···KAc, (c) Trp···KFm, and (d) Trp···KFAc based on the NMR chemical shifts in Figures 3 and 6.

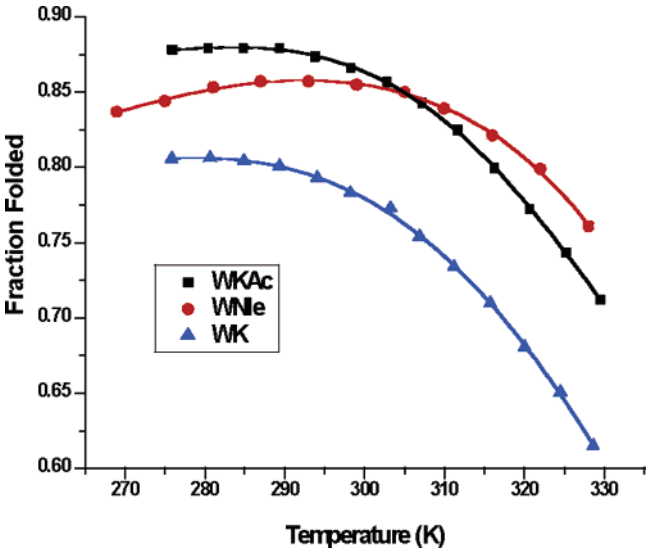

Figure 5. Thermal denaturation profiles of **WK**, **WKAc**, and **WNle** peptides as determined by NMR. The fraction folded was determined from the Gly splitting. Error is ±0.5 K in temperature and ±1% in fraction folded. Conditions: 50 mM NaOAc-d_3 buffer, pD 4.0 (uncorrected).

NH in KAc versus the ϵ-CH_2 in Lys likely arises from differences in the desolvation cost for an amide relative to an ammonium group. Indeed, the calculated solvation energy for the Lys side chain is −66.2 kcal/mol, versus −8.1 kcal/mol for KAc.[19] Moreover, if the amide in KAc is stacked with the Trp residue, the NH group may still be able to hydrogen bond with water, such that there is minimal desolvation penalty, as has been proposed for the interaction of Arg with Trp.[20] In proteins, the stacked conformation for Asn and Gln side chains with aromatic residues is favored by a factor of about 2.5.[6d] Given that the Trp···KAc interaction is highly solvent exposed in this peptide model system, a stacked conformation is likely.

The thermodynamics of hairpin folding for **WKAc** are consistent with hairpin stabilization due to a polar−π interaction (Figure 5 and Table 1).[21] The driving force for folding of **WKAc** is similar to **WK**, with an enthalpic driving force for the folding and an associated entropic cost, despite the difference in charge. In contrast, comparison with a peptide containing a nonpolar alkyl side chain, **WNle**, at position X shows that the driving force folding of **WKAc** is much more enthalpically favorable than the nonpolar Nle side chain.[15b] This is consistent with significant interaction between the polarized amide NH and

(19) Solvation energies for the Lys and KAc side chains were calculated with Gaussian 03 (implicit water/IEFPCM model/HF:6-31g**). Gaussian 03, Frisch, M. J., et al., Gaussian, Inc., Wallingford CT, 2004. The full reference can be found in the Supporting Information.
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Table 1. Thermodynamic Parameters[a] for Folding at 298 K[10]

peptide	$\Delta H°$ (kcal/ mol)	$\Delta S°$ (cal/ mol K)	$\Delta C_p°$ (cal/ mol K)	reference
WK	−2.8 (0.03)	−6.8 (0.1)	−163 (3)	15a,b
WKAc	−2.91 (0.07)	−6.1 (0.2)	−250 (40)	this work
WNle	−0.85 (0.07)	−0.7 (0.2)	−170 (25)	15b

[a] Determined from the temperature dependence of the Gly chemical shift from 0 to 60 °C. Errors (in parentheses) are determined from the fit. Error is ±0.5 K in temperature and ±1% in fraction folded. Conditions: 50 mM NaOAc-d_3 buffer, pD 4.0 (uncorrected).

tryptophan in **WKAc** as seen in Figure 3b, and argues against a hydrophobic driving force alone.[22]

Effects of Acetyl Lysine Analogues. Acetyl lysine analogues, formyl lysine (KFm) and trifluoroacetyl lysine KFAc), were used to further examine the effects of acylation. KFm was investigated in order to determine the contribution of hydrophobic or van der Waals interactions, and KFAc was studied to examine electronic effects on the interaction with Trp interaction. With these analogues, subtle changes were detected in the geometry of the interaction, but there was little effect on stability and driving force for folding. **WKAc** and its analogues **WKFm** and **WKFAc** are approximately equistable at 298K: 87, 86, and 87% folded (± 1%), respectively, based on the glycine splitting (83, 82, and 83% ± 8% folded based on the average side chain Hα fraction folded).[18] This is supported by the consistency of the Hα shifts relative to random coil (Figure 6a). While these hairpins are of similar stabilities, comparison of the side chain upfield shifting profiles for KAc, FAc, and Fm indicates differences in the mode of interaction with Trp (Figures 4, 6b). The KFm side chain has a similar profile to the KAc side chain. Both are most upfield shifted at the amide NH position. However, the KFm has both polarized amide and aldehyde protons (Figure 7), both of which can interact favorably with the tryptophan ring. As a result, the upfield shifting is distributed more evenly over the two sites, while the KAc side chain has a definite preference for interacting with tryptophan through the amide position (Figure 4). The interaction of KFm with Trp is in agreement with predictions by Duan et al. from gas-phase calculations that a formamide−π interaction is stable over a range of geometries.[12b] In contrast, the KFAc side chain exhibits significant upfield shifting at the δ- and ϵ-CH_2 groups and very little shifting of the amide nitrogen, suggesting a shift in the preferred geometry (Figures 4, 6b). Indeed, it has an interaction profile more similar to lysine (Figure 6c).

The differences in interaction geometries can be elucidated through a comparison of electrostatic potential maps of the three side chains (KAc, KFm, and KFAc) as shown in Figure 7. Clearly the amide proton is the most polarized site on each of the three side chain analogues, with the carbonyl carbon on the KFAc analogue also highly polarized due to the inductive effects of the fluorines. However, the fluorines in the KFAc analogue may present both a steric and electronic barrier to interaction of the amide NH with the indole ring. The CF_3 group in KFAc is the largest of the groups on the carbonyl, thus obscuring the amide proton. Additionally, CF···π interactions have been shown to have weak repulsive character in fluoromethane−benzene model systems, lending some repulsive electronic

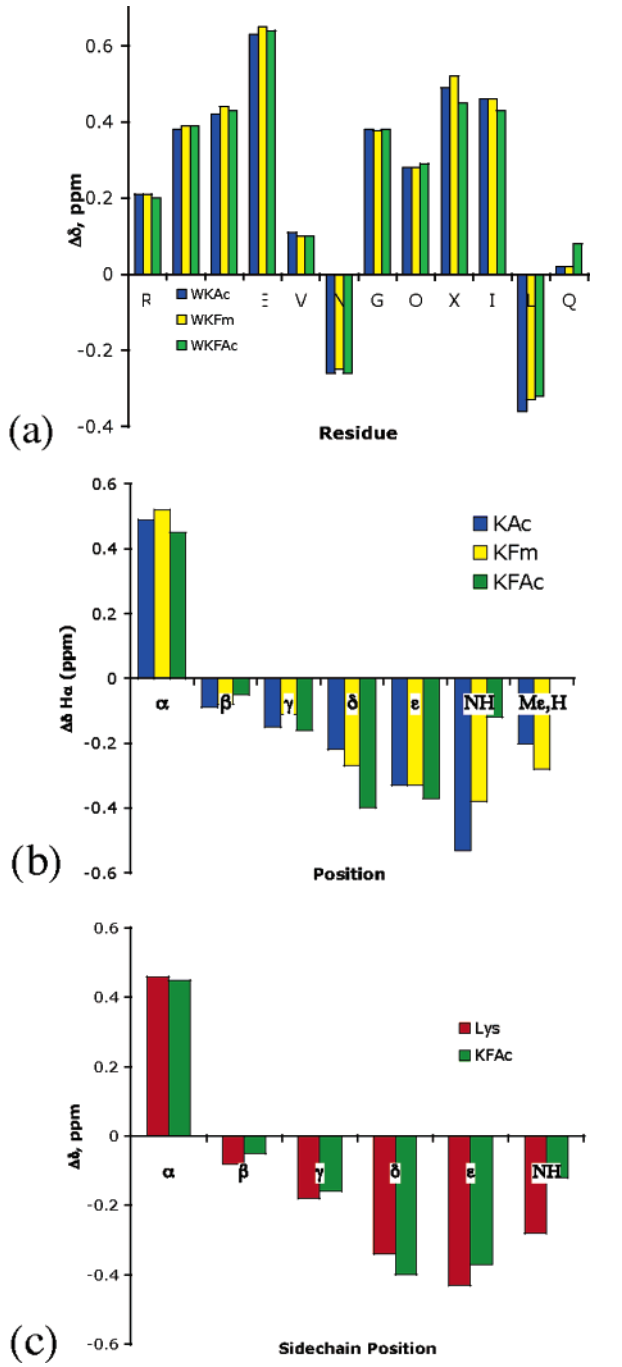

Figure 6. (a) **WKAc**, **WKFm**, and **WKFAc** αH shifts relative to random coil values. Glycine shifts reflect the splitting. (b) KAc, KFm, and KFAc side chain upfield proton shifts relative to random coil values. (c) Lys and KFAc side chain upfield proton shifts relative to random coil. Conditions: 298 K, 50 mM NaOAc-d_3 buffer, pH 4.0 (uncorrected), referenced to DSS.

character to the KFAc···Trp interaction.[23] Due to long-range inductive effects of the fluorines, the δ and ϵ positions of the KFAc side chain are more polarized than the corresponding positions in the KAc and KFm side chains (Figure 7). This, in combination with the electronic and steric repulsion of the trifluoro group, would explain the upfield shifting pattern seen in Figure 5c, where the most intense shifts are seen at the δ and ϵ positions of the KFAc side chain. This suggests that the interaction energy for the Trp···KFAc interaction can only be

(22) The difference in ΔC_p for WK and WKAc may also reflect differences in desolvation energy, although no clear trend is observed when a comparison to WNle is also made. Since a large, negative ΔC_p for folding is taken as evidence of a hydrophobic driving force for folding, the inconsistency between WKAc and WNle may have to do with differences in chain length.

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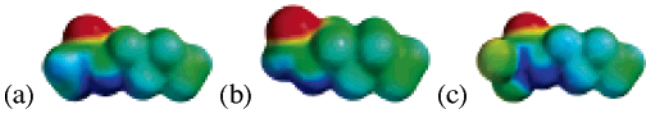

Figure 7. Electrostatic potential maps of amino acid side chains: (a) KAc, (b) KFm, (c) KFAc. Electrostatic potential maps generated with MacSpartan (HF/6-31g*; Isodensity value = 0.02; range = −30 to 30 kcal/mol).

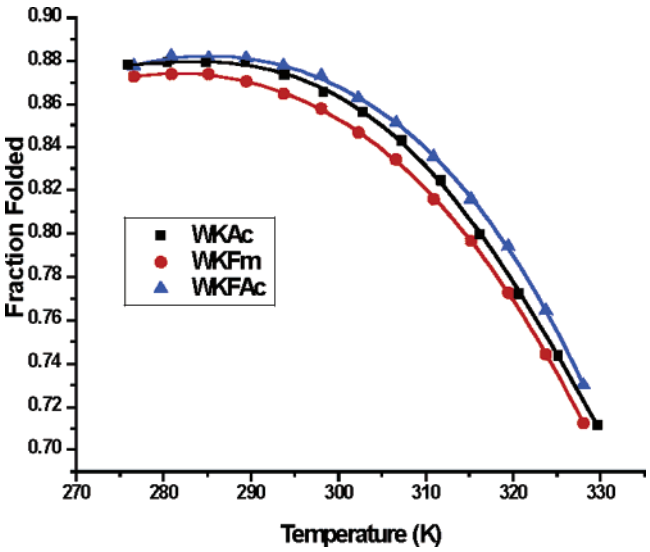

Figure 8. Thermal denaturation profiles of **WKAc**, **WKFm**, and **WKFAc** peptides as determined by NMR. The fraction folded was determined from the Gly splitting. Error is ±0.5 K in temperature and ±1% in fraction folded. Conditions: 50 mM NaOAc-d_3 buffer, pD 4.0 (uncorrected).

Table 2. Thermodynamic Parameters[a] for Folding at 298 K

peptide	$\Delta H°$ (kcal/ mol)	$\Delta S°$ (cal/ mol K)	$\Delta C_p°$ (cal/ mol K)
WKAc	−2.91 (0.07)	−6.1 (0.2)	−250 (40)
WKFm	−3.06 (0.03)	−6.7 (0.1)	−195 (30)
WKFAc	−2.68 (0.07)	−5.2 (0.2)	−220 (30)

[a] Determined from the temperature dependence of the Gly chemical shift from 0 to 60 °C. Errors (in parentheses) are determined from the fit.

maintained with a change in the geometry of interaction relative to Trp···KAc. This sort of subtle tuning of the interaction geometry has relevance to specificity in protein folding and protein−ligand interactions.

The thermal denaturation for each of the three peptides **WKAc**, **WKFm**, and **WKFAc** were found to be close to or within error (Figure 8), resulting in very similar values for $\Delta H°$, $\Delta S°$, and $\Delta C_p°$ (Table 2). Thus, although changing the sterics and electronics of the amide group influences the geometry of the interaction, there is very little effect on its driving force. This suggests a level of control of the interaction geometry that is not often observed with noncovalent interactions. Moreover, it provides insight into the subtle factors that may influence the folding of a protein into a single, low energy structure.

Conclusion

We have utilized a β-hairpin model system to investigate the nature of an amide−π interaction between Trp and an acetylated Lys residue and compared it to the cation−π interaction of Trp with the unmodified Lys. Our system demonstrates both the geometric and energetic effects of acetylation upon the Trp···Lys interaction. Surprisingly, while the geometry of the interaction changes upon acetylation, the magnitude of the interaction does not decrease, despite the loss of a positive charge. NMR chemical shifts indicate that the amide NH is in close proximity to the face of the aromatic ring, rather than between the ϵ-CH_2 and Trp that is observed with the unmodified Lys. The change in geometry is likely due to differences in desolvation costs for the Lys and KAc side chains. The thermodynamics for folding indicate that the amide−π interaction contains significant polar−π character. Thus, both amide−π and cation−π interactions consist of a sum of a number of components, including charge−quadrupole (for cation−π), dipole−quadrupole, and quadrupole−quadrupole interactions (for amide−π), as well as dispersion forces and hydrophobic effects.[24] The primary difference between the two types of interactions is that conversion of the ammonium group to an amide changes the desolvation cost of the nitrogen group, such that the NH of the amide can favorably interact directly with the aromatic ring, which is too costly for the ammonium group. Thus, the terms "cation−π" and "amide−π" are useful as general terms that emphasize the importance of quadrupole interactions rather than simple hydrophobic effects alone, but by no means do these terms indicate that quadrupole interactions are the only contributor to these noncovalent interactions.

Acetylated lysine analogues KFm and KFAc have further elucidated our understanding of this interaction through manipulation of both electronic and sterics of the Lys side chain. These changes in sterics and electronics result in subtle changes in the geometery of interaction which are required to maintain the interaction energy, indicating the fine-tuning that is possible in such noncovalent interactions. This has significance for understanding specificity in protein folding as well as in protein−ligand interactions and may provide useful insight for medicinal chemists. In summary, these studies provide insight into the specific recognition of acetylated lysine that plays a significant biological role in chromatin condensation via the "histone code" as well as the general role of amide−π interactions in protein structure and function.

Experimental Section

Synthesis of Fmoc-Trifluoroacetyl Lysine. Fmoc-lysine (0.384 g; 0.97 mmol) was dissolved in THF (10 mL) and cooled in an ice bath to 0 °C. DIPEA (168 μL, 0.97 mmol) was added dropwise to the cold solution. Trifluoroacetic anhydride (162 μL, 1.2 mmol) was added in one portion, and the resulting solution was allowed to warm to room temperature over 20 min. The reaction was followed by TLC (10:1 CH_2Cl_2:MeOH). The resulting solution was rotovapped to dryness, taken up into ethyl acetate, and washed with water and brine. The organic layer was dried with sodium sulfate, and the solvent was removed in vacuo giving a clear oil. Further purification with silica gel chromatography (10:1 CH_2Cl_2:MeOH) gave 0.405 g of a clear oil (0.87 mmol; 90% yield). ESI-MS: calculated = 464.156; actual = 464.2 NMR (600 MHz, $CDCl_3$): 7.83 (d, 2H); 7.60 (d, 2H); 7.42 (dd, 2H); 7.33 (dd, 2H); 6.59 (s, 1H); 5.415 (d, 1H); 4.44 (m, 2H); 4.23 (m, 1H), 3.38 (m, 2H); 1.95−1.47 (m, 6H).

Synthesis of Fmoc-Formyl Lysine. Formic acid (534 μL, 14 mmol) was added dropwise to acetic anhydride (1.086 mL, 11.4 mmol) at 0 °C, followed by gentle heating (60 °C, 2 h).[25] The resulting mixture was cooled to 0 °C in an ice bath. Fmoc-Lys (0.384 g; 1.0 mmol) was taken up into 5 mL of THF and added dropwise to the mixed anhydride solution. The resulting solution was allowed to stir overnight at room temperature. After removal of the solvent *in vacuo*, the solution was taken up into ethyl acetate (25 mL) and washed with water (25 mL)

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and brine (25 mL). The organic layer was dried with sodium sulfate and the solvent removed in vacuo, resulting in a clear oil (0.390 g; 98% yield; 0.98 mmol). ESI-MS: calculated = 396.169; actual = 396.2 NMR (600 MHz, $CDCl_3$): 8.08 (s, 1H), 7.74 (d, 1H), 7.58 (d, 2H), 7.38 (dd, 2H), 7.28 (dd, 2H), 6.07 (s,1H); 5.71 (s, 1H); 4.38 (m, 2H); 4.19 (m, 1H), 3.26 (m, 2H); 1.25−1.9 (m, 6H).

Peptide Synthesis. Fmoc-Lys(Ac) was purchased from Bachem. Fmoc-trifluoroacetyl lysine (Fmoc-KFAc) and Fmoc-formyl lysine (Fmoc-KFm) were synthesized as described above. The synthesis of all peptides was performed on an Applied Biosystems Pioneer peptide synthesizer using Applied Biosystems PEG-PAL amide resin. Peptides were synthesized on a 0.1 or 0.07 mmol scale. All amino acids with functionality were protected during synthesis as follows: Arg(Pbf), Asn-(trt), Lys(Boc), Orn(Boc), Gln(trt), Trp(Boc), and Glu(tBu). Coupling reagents were HBTU/HOBt. Noncommercially available amino acids were coupled by hand. The N-terminus was acetylated for all peptides with a solution of 5% acetic anhydride and 6% 2,6-lutidine in DMF. Cleavage conditions removed all side chain protection with a cocktail of 90% TFA/5% Triisopropylsilane/5% H_2O. Peptides were purified by RP-HPLC on a C18 column. Peptides were purified with a gradient of A and B (A: 95% H_2O/5% ACN with 0.1% TFA, B: 95% ACN/ 5% H_2O with 0.1% TFA). Once purified, peptides were lyophilized to powder and characterized by ESI-TOF mass spectroscopy and NMR.

NMR Spectroscopy. NMR samples were made in concentrations of approximately 1 mM and analyzed on a Varian Inova 600 MHz spectrometer. Samples were dissolved in D_2O buffered to pD 4.0 (uncorrected) with 50 mM NaOAc-d_3 and referenced to DSS. Amine and amide resonances were assigned in 60% H_2O solutions. 1D NMR spectra were collected using 32K data points and between 8 and 64 scans using a 1−3 s presaturation. All 2D NMR experiments used pulse sequences from the Chempack software including TOCSY, DQCOSY, gCOSY, and NOESY. 2D NMR scans were taken with 16−64 scans in the first dimension and 64−256 scans in the 2nd dimension. All spectra were analyzed using standard window functions (sinebell and Gaussian). Mixing times of 0.5 s were used in the NOESY spectra. Assignments were made using standard methods as described by Wüthrich.[26] Thermal denaturations were run in duplicate, with standard deviations of less than 0.005 ppm. Samples were allowed to equilibrate for 7 min at each temperature before the measurement was taken. Temperature calibrations were made using methanol and ethylene glycol standards.

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Supporting Information Available: Control peptides, chemical shift assignments, NOEs, and thermal denaturation data are available in Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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