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Arginine Methylation in a β-Hairpin Peptide: Implications for Arg$-\pi$ Interactions, $\Delta C_p°$, and the Cold Denatured State

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Abstract: Arginine methylation is a common post-translational modification that plays a role in many cellular processes through mediation of protein−protein interactions. There is still a dearth of structural information as to its role in mediating such interactions, but the available data suggest a possible role of cation$-\pi$ interactions in the recognition of methylated arginine. Hence, the effect of arginine methylation on its interaction with tryptophan has been investigated within the context of a β-hairpin peptide. Arginine methylation was found to enhance the stacking interaction between the cationic guanidinium functionality of arginine and the indole ring of tryptophan, resulting in structural stabilization of the hairpin. Thermodynamic analysis reveals more favorable entropy of hairpin folding with arginine methylation, a more negative change in heat capacity for folding, and a modest decrease in enthalpic driving force. This is consistent with enhanced stacking and hydrophobic interactions through increased surface area of the guanidinium moiety and greater delocalization of positive charge. In addition, these peptides exhibit significant cold denaturation, which can be accounted for by the inclusion of an expression of temperature-dependent ΔC_p in the thermodynamic analysis.

Introduction

Arginine methylation is a common post-translational modification that has recently been identified as playing a significant role in the regulation of cellular processes.[1] Both arginine (Arg) mono- and dimethylation have been shown to play a role in cell signaling through mediation of protein−protein interactions.[2] In particular, methylated arginines have been shown to function, in concert with other post-translational modifications, in chromatin restructuring and transcriptional activation.[1b] There is still little structural information on record for methylated arginine, and so little is known about how such a subtle change in structure may mediate biomolecular recognition and signaling. However, several recent studies of proteins containing dimethylarginine (DMA), as well as recent structural information on the role of the related post-translational modification trimethyllysine (KMe3) in protein−protein interactions, suggest a possible mechanism for recognition of the modified amino acid: the specific recognition of methylated arginine may depend on its interaction with aromatic rings (Figure 1). For example, the Tudor domain, a common 60 amino acid protein domain with highly conserved tryptophan, tyrosine, and phenylalanine residues, has been shown to bind preferentially to symmetrically methylated arginine-containing proteins.[3] In the two examples of methylated arginine in the Protein Data Bank, it is found to stack with a tryptophan residue in a histone/chromodomain protein complex (Figure 1b)[4] and with an iron-containing heme (not shown).

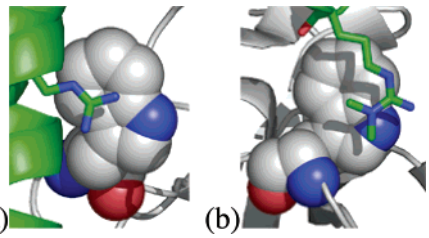

Figure 1. (a) Arg−Trp pair in typical cation$-\pi$ geometry (human growth hormone and receptor complex: 3HHR).[5] (b) DMAa−Trp pair from histone H3-chromodomain complex (2B2U).[4]

There is significant precedence for Arg stacking with aromatic residues. In surveys of protein crystal structures, cation$-\pi$ interactions are often observed between the guanidine groups of arginine side chains and the aromatic rings of tyrosine, tryptophan, and phenylalanine (Figure 1a).[6] Gallivan and Dougherty's survey of 593 protein crystal structures found that about 74% of arginine residues are in close proximity to an aromatic side chain, compared to 43% for Lys.[6d] This statistical preference is due to arginine's unique ability to interact with

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aromatic rings with a hybrid of the cation−π and π−π stacking interactions, which lends both favorable dispersion and electrostatic character to the interaction without a significant desolvation penalty. Additional surveys of protein structures have identified a specific preference for the stacking of arginine residues with the six-membered portion of the tryptophan indole ring.[7] Cation−π interactions involving arginine have been identified and investigated within the context of protein structure[6,8] and ligand binding,[9] protein−protein[10] and protein−nucleotide interactions,[11] and peptide folding[12,13] and in model receptor systems.[14] As with Lys, methylation of Arg may significantly enhance the magnitude of the Arg−π interaction while also making the residue more hydrophobic, thus providing a mechanism to mediate biomolecular recognition.

To probe this possibility, we have investigated the interaction between Trp and symmetrically or asymmetrically dimethylated arginine residues (DMAs and DMAa, respectively) within the context of a β-hairpin peptide (Figure 2). This system has previously been shown to be an excellent model system for studying noncovalent interactions in an aqueous environment.[13,15,16] We find that, in comparison to the Arg···Trp interaction, methylation of arginine significantly enhances peptide stability while maintaining the stacked geometry with tryptophan. Thermodynamic analysis of hairpin folding indicates that methylation of Arg results in a decreased entropic penalty for folding with a concomitant decrease in enthalpic driving force. Furthermore, our analysis suggests that inclusion of a temperature-dependent ΔC_p is necessary in some cases for a full accounting of the observed cold denaturation. These results suggest that the enhanced interaction of methyl arginine with an aromatic ring may be a key driving force in the mediation of protein−protein interactions by proteins containing methylated arginine.

Results and Discussion

Design, Synthesis, and Characterization. To study the Trp−dimethylarginine (DMA) interactions, we utilized the same β-hairpin peptide system that we had previously used to investigate Trp···Lys, Trp···Arg, and Trp···KMe3 interactions (Figure 2).[13,15,16] Fmoc-DMAs and Fmoc-DMAa were purchased from Bachem and incorporated into the peptide via standard Fmoc solid-phase peptide synthesis. The peptides were characterized by mass spectrometry and NMR as reported previously.[13] α-Hydrogen (Hα) chemical shifts, glycine splitting, amide shifts, and NOEs were used to characterize the hairpins and their respective stabilities.[17] The Hα chemical shifts of the hairpins relative to random coil values (Figure 3a) indicate the degree of β-sheet structure at each position along the strand, where downfield shifting of greater than 0.1 ppm is taken to indicate a β-sheet structure.[18] The magnitude of glycine splitting has also been shown to be a good indicator of overall hairpin stability.[17] The extent of folding can be quantified from both the Hα chemical shift and Gly splitting through comparison to a cyclic peptide which represents the fully folded state (see Supporting Information). Furthermore, NOEs between cross-strand pairs of side chains were investigated to confirm β-hairpin formation as well as the specific side chain−side chain interaction between Trp and DMA (see Supporting Information). Last, upfield shifting of the DMA side chain provides information about its proximity to the face of the Trp residue.

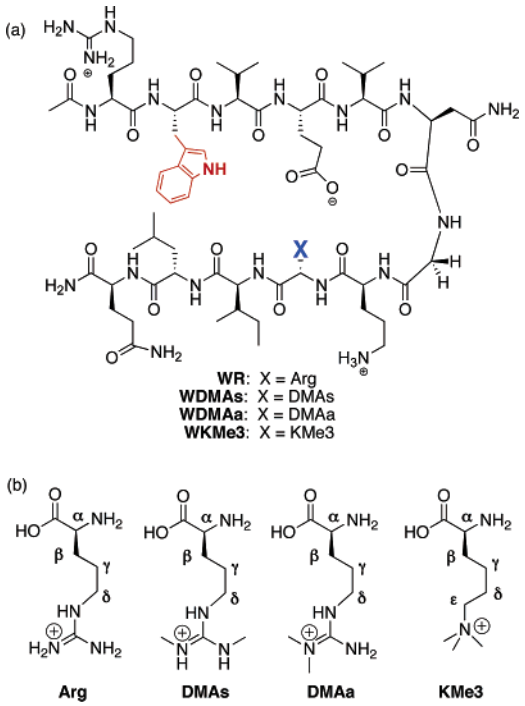

Figure 2. (a) β-Hairpin peptides containing arginine (Arg), symmetrically and asymmetrically dimethylated Arg (DMAs and DMAa, respectively), and trimethylated Lys (KMe3); peptides are referred to in the text by their residues in position 2 (Trp) and position 9 (X). (b) Amino acids at position X.

Effect of Methylation on Hairpin Stability and Structure. Incorporation of DMAs or DMAa results in an increase in hairpin stability relative to the parent peptide. This is demonstrated by a measurable increase in the Hα shifts relative to random coil, as shown in Figure 2a. Based on the glycine splitting (Table 1), fraction folded of **WDMAs** is 94% and **WDMAa** is 93%, versus 84% for the peptide **WR** at 298 K, indicating that both symmetric and asymmetric dimethylation enhance hairpin stability similarly, by about 0.6 kcal/mol relative

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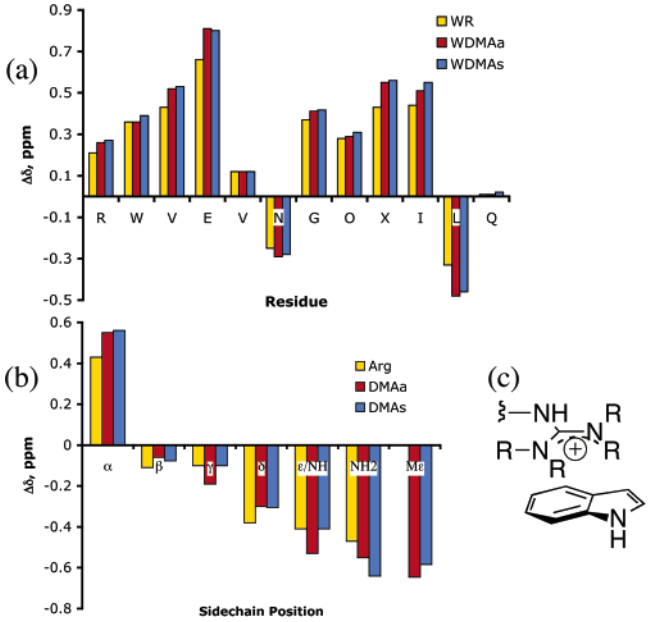

Figure 3. (a) **WR**, **WDMAa**, and **WDMAs** Hα shifts relative to random coil values. Glycine shifts reflect the splitting. (b) **Arg**, **DMAa**, and **DMAs** side chain upfield proton shifts relative to random coil values. Conditions: 298 K, 50 mM NaOAc buffer, pH 4.0 (uncorrected), referenced to DSS. (c) Proposed geometry for the Trp···DMA interaction.

Table 1. Fraction Folded and Stability of Hairpins at 298 °C

	% folded		
peptide	Gly[a]	Hα[b]	ΔG, kcal/mol[c]
WDMAs	94(1)	93(3)	−1.6(−1.5)
WDMAa	93(1)	90(6)	−1.5(−1.3)
WR	84(1)	78(7)	−1.0(−0.7)

[a] Error is ±1%, as determined from the error in chemical shift. [b] Percent folded from Hα shifts is the average of the values from residues 2−5 and 8−11, excluding the turn residues and the termini. The standard deviation is in parentheses. [c] Determined from the Gly splitting; values in parentheses are from the Hα data. Error is ±0.05 kcal/mol, as determined from the error in the chemical shift.

to **WR**. Values determined from the Hα chemical shifts are in good agreement with the values determined from the Gly splitting (Table 1). Additionally, the chemical shifts for the arginine side chains show enhanced upfield shifting upon methylation (Figure 3b). The magnitude of upfield shifting across the guanidinium functionality is consistent with a stacking interaction between arginine and tryptophan that is enhanced by methylation (Figure 3c). Interestingly, for DMAs, the interaction is shifted toward the $NHCH_3$ groups, whereas for DMAa, there is similar upfield shifting at the ϵ-NH, NH_2, and methyl groups, suggesting a difference in the orientation of stacking for the two different DMA residues.

Interaction Energies. Double mutant cycles were performed to determine the magnitude of the side chain−side chain interaction in isolation, using the same substitutions at positions 2 and 9 that were reported previously for the parent peptide, **WR**: Val was substituted for Trp at position 2, and Ser was substituted for DMA at position 9 (Figure 4).[13] Double mutant cycles are necessary because in each of the single mutants the Arg···Trp interaction is disrupted, but other changes occur as well, such as the β-sheet propensities of the substituted residues. A double mutant containing both Val and Ser corrects for any of the unintentional changes, such that the magnitude of the DMA···Trp interaction can be determined as shown in Figure

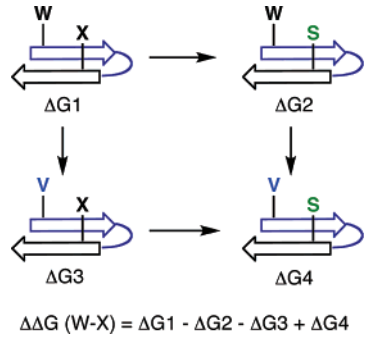

Figure 4. Double mutant cycle to measure the interaction energy between Trp and residue X.

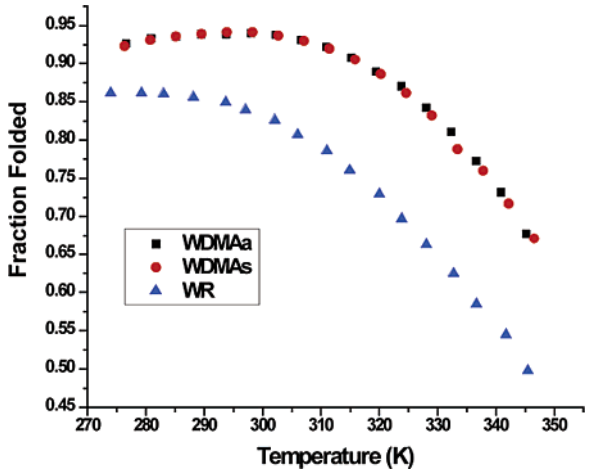

Figure 5. Thermal denaturation of peptides **WR, WDMAs**, and **WDMAa** as measured by the temperature dependence of the Gly splitting.

4. This gave energies of −1.0(±0.1) kcal/mol for the cation−π interaction with both DMAs and DMAa. Thus, methylation enhances the Arg···Trp interaction by about a factor of 2.[13] Interestingly, the interaction between Trp and DMA is equal to that of Trp and KMe3.[16]

Effect of Arg Methylation on Thermodynamic Analysis. The thermodynamic driving force for hairpin folding was investigated by performing thermal denaturation studies on each of the peptides, followed by NMR (Figure 5). Methylation of Arg resulted in a substantial increase in thermal stability of the hairpin, with no observable difference between **WDMAs** and **WDMAa**. The temperature of maximum stability (T_{max}) for **WR** is about 275 K, versus about 295 K for **WDMAs** and **WDMAa**, amounting to a 20 K increase. Moreover, **WDMAa** and **WDMAs** exhibit cold denaturation below 295 K, which is related to the change in buried hydrophobic surface area and is typically associated with a hydrophobic driving force for folding.

We fit the data to extract $\Delta H°$, $\Delta S°$, and $\Delta C_p°$ of folding using the method reported by Searle (eq 1), which assumes a temperature-independent ΔC_p:[17]

$$\text{Fraction Folded} = [\exp(x/RT)]/[1 + \exp(x/RT)] \quad (1)$$

where $x = [T(\Delta S°_{298} + \Delta C°_p \ln(T/298)) - (\Delta H°_{298} + \Delta C°_p(T - 298))]$. This expression has been shown to fit non-cold-denatured β-hairpin data reasonably well, but for **WDMAa** and **WDMAs**, the fit to the cold-denatured portion of the curve was not as good, based on visual inspection (Figure 6b,c). Andersen has recently shown that the cold-denatured state of a β-hairpin

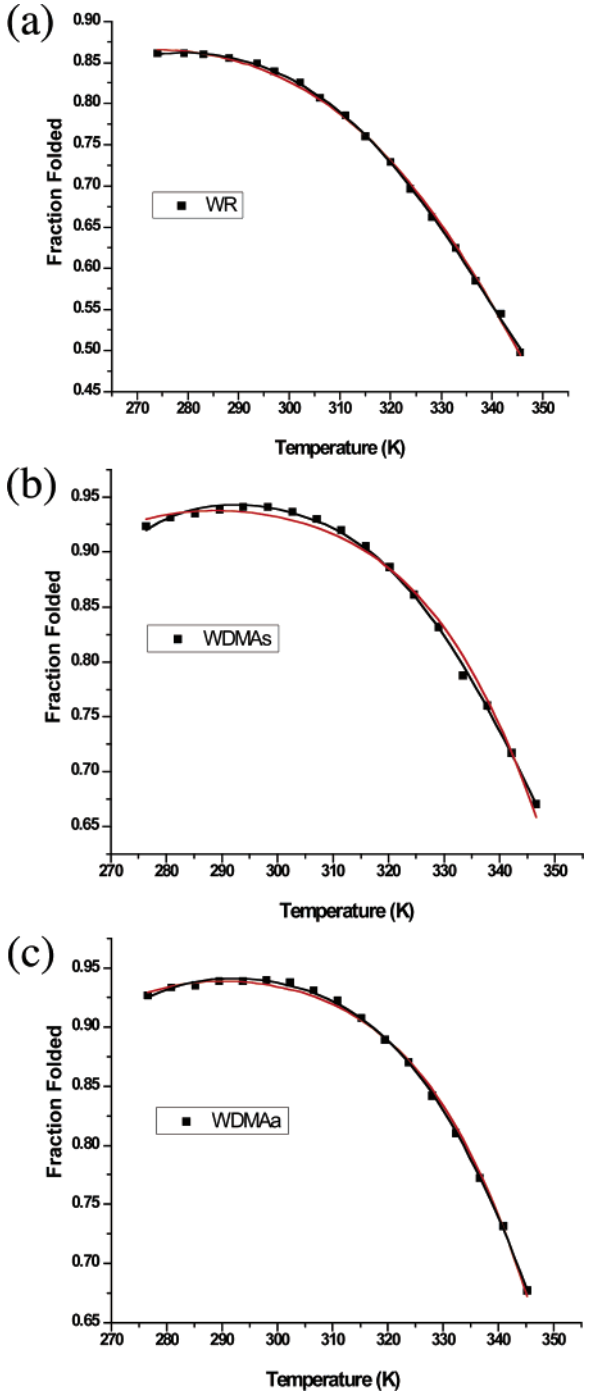

Figure 6. Fitting of thermal denaturation profiles of (a) **WR**, (b) **WDMAs**, and (c) **WDMAa** peptides using eq 1 with a temperature-independent ΔC_p (red line) and eq 9, which allows ΔC_p to vary with temperature (black line). The fraction folded was determined from the Gly splitting. Error is ±0.5 K in temperature and ±1% in fraction folded. Conditions: 50 mM NaOAc-d_4 buffer, pD 4.0 (uncorrected).

differs from the heat-denatured state and that it retains some hydrophobic interactions.[19] The change in heat capacity upon folding, $\Delta C_p°$, is an indicator of the importance of hydrophobic contacts to the folding event (i.e., side chain interactions), where a negative $\Delta C_p°$ of folding (or a positive $\Delta C_p°$ of unfolding) is an indication of hydrophobic clustering within the folded state of the hairpin.[20] If there are differences between the cold- and heat-denatured states, such as residual structure, then the ΔC_p would be expected to vary with temperature. Hence, we considered the possibility that the ΔC_p is not temperature-independent across the entire temperature range. We re-derived the Searle equation assuming a temperature-dependent ΔC_p, using an empirical expression for the temperature dependence of the heat capacity as shown below:[21]

$$C_{p,m} = a + bT + c/T^2 \quad (2)$$

$$\Delta H° = \Delta H°_{298} + \int C_{p,m}\,dT \quad (3)$$

$$\int C_{p,m}\,dT = a(T - 298) + (b/2)(T^2 - 298^2) - c(1/T - 1/298) \quad (4)$$

$$\Delta H° = \Delta H°_{298} + a(T - 298) + (b/2)(T^2 - 298^2) - c(1/T - 1/298) \quad (5)$$

$$\Delta S° = \Delta S°_{298} + \int (C_{p,m}/T)\,dT \quad (6)$$

$$\int (C_{p,m}/T)\,dT = a\ln(T/298) + b(T - 298) - (c/2)(1/T^2 - 1/298^2) \quad (7)$$

$$\Delta S° = \Delta S°_{298} + a\ln(T/298) + b(T - 298) - (c/2)(1/T^2 - 1/298^2) \quad (8)$$

Substituting eqs 5 and 8 into eq 1 gives the following expression:

$$\text{Fraction Folded} = [\exp(x/RT)]/[1 + \exp(x/RT)] \quad (9)$$

where $x = T(\Delta S°_{298} + a\ln(T/298) + b(T - 298) - (c/2)(1/T^2 - 1/298^2)) - (\Delta H°_{298} + a(T - 298) + (b/2)(T^2 - 298^2) - c(1/T - 1/298))]$.

Using eq 9, which allows ΔC_p to vary with temperature, we re-analyzed the data. The effect on **WR** was first investigated, as this peptide does not exhibit noticeable cold denaturation. Fitting of its thermal denaturation data is marginally better using eq 9, based on visual inspection (Figure 6a). We performed an *F* test to determine whether the better fit is significant and found that it is significant within 95% confidence limits.[22] Nonetheless, eq 9 gives values for $\Delta H°$ and $\Delta S°$ within error of those from eq 1 in this case, with a somewhat more negative $\Delta C_p°$ (Table 2). This indicates that, in the absence of noticeable cold denaturation, the assumption that ΔC_p is independent of temperature is generally reasonable, and the simplified equation reported by Searle is satisfactory.

Comparison of fits for **WDMAs** and **WDMAa** with eqs 1 and 9 indicates that, in these cases, in which cold denaturation is observed, a noticeably better fit is obtained with eq 9 (Figure 6b,c). This was verified using the *F* test. Nonetheless, fitting with eq 9 has no significant effect on the values of $\Delta H°$ and

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Table 2. Thermodynamic Parameters[a] for Hairpin Folding at 298 K Peptides **WR**, **WDMAs**, and **WDMAa** Using Eq 1 (Temperature-Independent ΔC_p) and Eq 9 (Temperature-Dependent ΔC_p)

	eq 1			eq 9		
peptide	$\Delta H°$	$\Delta S°$	$\Delta C_p°$	$\Delta H°$	$\Delta S°$	$\Delta C_p°$
WR	−3.6(0.1)	−8.8(0.3)	−152(6)	−3.7(0.1)	−9.1(0.2)	−193(29)
WDMAs	−2.4(0.3)	−2.9(1.2)	−260(18)	−2.4(0.2)	−2.6(0.8)	−409(61)
WDMAa	−2.2(0.2)	−2.1(0.6)	−280(10)	−2.3(0.1)	−2.1(0.4)	−355(53)

[a] Determined from the temperature dependence of the Gly chemical shift from 0 to 80 °C. Units are $\Delta H°$, kcal/mol; $\Delta S°$, cal/mol·K; $\Delta C_p°$, cal/mol·K. Errors (in parentheses) are determined from the fit. Error for $\Delta C_p°$ values from eq 9 estimated at 15%.

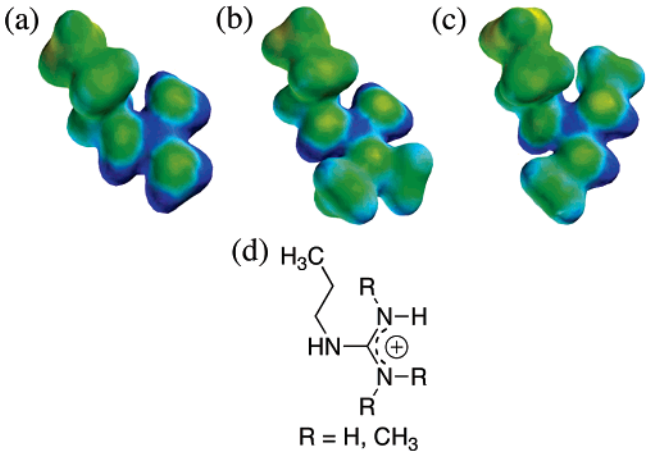

Figure 7. Electrostatic potential maps of side chains: (a) Arg, (b) DMAa, and (c) DMAs. (d) Structure of the Arg side chain indicating its orientation in the electrostatic potential maps. Electrostatic potential maps were generated with MacSpartan: HF/6-31g*; isodensity value = 0.02; range = 50 (red, electron rich) to 200 kcal/mol (blue, electron poor).

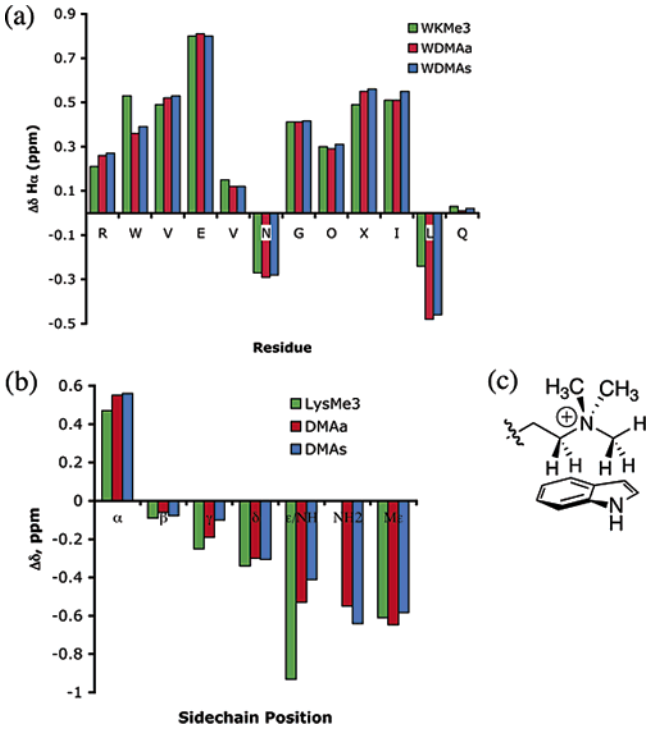

Figure 8. (a) **WDMAa**, **WDMAs**, and **WKMe3** Hα shifts relative to random coil values. Glycine shifts reflect the splitting. (b) DMAa, DMAs, and KMe3 side chain upfield proton shifts relative to random coil values. Conditions: 298 K, 50 mM NaOAc-d_4 buffer, pD 4.0 (uncorrected), referenced to DSS. (c) Proposed geometry for the Trp···KMe3 interaction.

$\Delta S°$ (Table 2). In each case, the errors for $\Delta H°$ and $\Delta S°$ are smaller using eq 9, and the values for $\Delta C_p°$ are more negative than with eq 1. This is likely due to a better accounting of the cold-denatured state.[20]

Comparison of the thermodynamics of hairpin folding for **WDMAs** and **WDMAa** obtained from eq 9 indicates that the two hairpins are very similar, with a reduced enthalpic driving force for folding relative to **WR** and with a lower entropic cost for folding, as well as a more negative $\Delta C_p°$ (Table 2). These data suggest that methylation of Arg results in a reduced electrostatic driving force for interaction with Trp and an enhanced hydrophobic component.[20]

Examination of the electrostatic potential maps of arginine and methylated arginine side chains provides additional insight into the observed effects (Figure 7). Upon methylation, two changes are apparent: the surface area of the guanidinium group is significantly increased and the positive charge (indicated by the blue regions) is further distributed across the methyl functionality.[23] This distribution of charge is expected to diminish the electrostatic interaction between the arginine guanidinium and the tryptophan indole ring, resulting in a reduced enthalpic driving force, as is observed. The increased surface area may increase the dispersion forces between the two systems, which would be enthalpically favorable, counteracting the effect of the greater charge distribution. However, it will also make the residue more hydrophobic, such that folding would be more entropically favorable. The facts that the entropy of folding becomes more favorable upon methylation and that the $\Delta C_p°$ for folding becomes more negative argue in favor of an increased hydrophobic effect relative to the parent Arg··· Trp interaction (Table 2).

Comparison to Trimethylated Lysine. The peptide **WKMe3** has been previously reported to be a highly thermally stable β-hairpin due to a methylation-enhanced Lys···Trp interaction.[16] Its comparison to the dimethylated arginine peptides is particularly interesting, since trimethylation of lysine is also a common post-translational modification and has been shown to induce binding to an aromatic pocket in the protein chromodomain.[24] While the DMA- and KMe3-containing peptides are of similar stability at 298 K (Figure 8a), they have distinctly different modes of interaction with tryptophan. The trimethylated lysine interacts with tryptophan primarily by packing its ϵ-CH_2 and terminal methyl groups into the face of the indole ring, with

(23) For example, on the central carbon of the guanidinium group, the charge decreases from = +1.16 for Arg to +0.54 for DMAs and +0.76 for DMAa, as determined from the Merz−Kollman atomic charges. See Supporting Information.

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the predominant site for interaction at the ϵ-CH_2 group, as evidenced by the upfield shifting of the ϵ-CH_2 by nearly 1 ppm (Figure 8b).[16] In contrast, the dimethylated arginine derivatives undergo stacking interactions, as evidenced by the relatively even distribution of proton upfield shifting across the guanidinium and methyl groups (Figure 8b).

Both sets of peptides demonstrate high thermal stability, with melting temperatures (T_m) greater than 75 °C, although NMR studies indicate that **WKMe3** remains more folded at high temperatures (Figure 9). Since **WKMe3** exhibits significant cold denaturation, we re-fit its thermal denaturation data using eq 9, and we also re-fit the parent peptide **WK** for comparison.[25] In the case of **WK**, which shows no cold denaturation, the fit with eq 9 was no better than the fit with eq 1 according to the *F* test (Figure 9b). However, a better fit was obtained for **WKMe3** with eq 9 than with eq 1 (Figure 9c), as was found with **WDMAa** and **WDMAs**. Interestingly, in this case the temperature-dependent fit resulted in variation of the values for $\Delta H°$ and $\Delta S°$, but with no change in $\Delta C_p°$ (Table 3). This may be because eq 1 appears to overestimate the cold denaturation in this case.

Whereas methylation of Arg has a modest effect on $\Delta H°$ and $\Delta S°$ of hairpin folding, methylation of Lys has a more considerable effect, resulting in a less favorable enthalpy of folding and a more favorable entropy (Tables 2 and 3).[16,25] The more favorable entropy of folding for WKMe3 can be explained by the greater conformational freedom of the KMe3−Trp interaction versus that of a DMA−Trp interaction, where the rotor-like KMe3 has more sites of interaction with planar Trp than DMA, which is confined primarily to a stacked orientation with Trp in the folded state. Additionally, $\Delta C_p°$ of folding, which is generally recognized as a better indicator of the hydrophobic driving force in proteins than $\Delta S°$,[20] is more favorable for DMA than for KMe3. The greater hydrophobic driving force for the DMA···Trp interaction over the KMe3···Trp interaction is consistent with the electrostatic potential maps (Figures 6 and 10) and the calculated Merz−Kollman atomic charges (see Supporting Information), which both show greater polarization of the KMe3 methyl hydrogens versus the DMA methyl hydrogens. Another likely reason for the more favorable $\Delta C_p°$ of folding for DMA may be differences in the solvent-accessible surface area of KMe3 and DMA in the folded state. Finally, the greater enthalphic driving force of the DMA···Trp interaction versus that of KMe3 is likely due to the π-system of the guanidinium functionality, which results in less charge distribution onto the methyl groups (see Figures 6 and 10) and lends an additional $\pi-\pi$ stacking component to the interaction with Trp. [6d,13]

Conclusion

Using a β-hairpin model system, we have found that methylation of Arg enhances its interaction with a neighboring aromatic residue. The interaction involves the stacking of DMA residue with the face of the Trp side chain. The interaction provides −1.0 kcal/mol in stability to the hairpin, about twice as much as that of the unmethylated Arg and equal to that of the interaction of Trp with KMe3. The methylation of Arg results in a decreased enthalpic driving force for folding with a concomitant decrease in entropic cost, consistent with an increased hydrophobic component to the Arg···Trp interaction. Inclusion of the temperature-dependent ΔC_p term improves the thermodynamic analysis for peptides exhibiting cold denaturation and may better account for hydrophobic interactions in the cold-denatured state. It is noteworthy that such subtleties in ΔC_p are observable in this system. Indeed, this system may

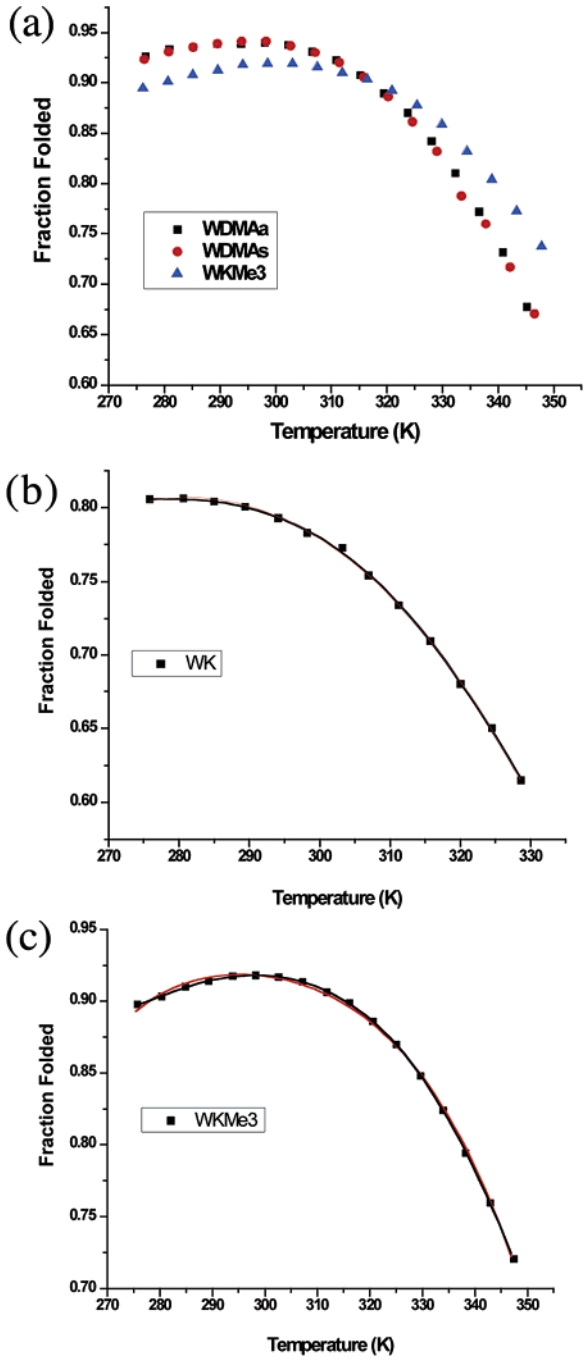

Figure 9. (a) Comparison of thermal denaturation profiles of **WDMAa**, **WDMAs, and WKMe3** peptides. (b,c) Fitting of the thermal denaturation profiles of **WK** and **WKMe3** using eq 1 with a temperature-independent ΔC_p (red lines) and eq 9, which allows ΔC_p to vary with temperature (black lines). 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_4 buffer, pD 4.0 (uncorrected).

(25) The values for $\Delta H°$, $\Delta S°$, and $\Delta C_p°$ obtained for **WKMe3** with eq 1 differ slightly from those reported in ref 14 because the data here come from the average of four runs rather than the average of two runs in ref 14. Nonetheless, the difference in fraction folded in any one data point in the thermal denaturation from different data sets was less than 1%.

Table 3. Thermodynamic Parameters[a] for Hairpin Folding at 298 K for Peptides **WK** and **WKMe3** Using Eq 1 (Temperature-Independent ΔC_p) and Eq 9 (Temperature-Dependent ΔC_p)

	eq 1			eq 9		
peptide	$\Delta H°$	$\Delta S°$	$\Delta C_p°$	$\Delta H°$	$\Delta S°$	$\Delta C_p°$
WK	−2.8(0.1)	−6.7(0.1)	−166(4)	−2.6(10.1)	−6.2(0.2)	-182(27)
WKMe3	−0.6(0.1)	+2.7(0.3)	−237(5)	−0.1(0.1)	+4.5(0.3)	−243(4)

[a] Determined from the temperature dependence of the Gly chemical shift from 0 to 80 °C. Units are $\Delta H°$, kcal/mol; $\Delta S°$, cal/mol·K; $\Delta C_p°$, cal/mol·K. Errors (in parentheses) are determined from the fit. Error for $\Delta C_p°$ values from eq 9 estimated at 15%.

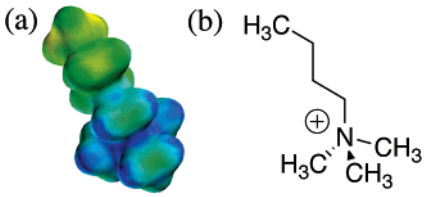

Figure 10. (a) Electrostatic potential map of the KMe3 side chains. (b) Structure KMe3 side chain indicating its orientation in the electrostatic potential map. The electrostatic potential map was generated with MacSpartan: HF/6-31g*; isodensity value = 0.02; range = 50 (red, electron rich) to 200 kcal/mol (blue, electron poor).

prove to be a useful model system for addressing questions about the role of heat capacity in protein folding.

In comparison to KMe3, DMA has less charge dispersion onto the methyl groups and can interact with a larger portion of the Trp indole ring, but with fewer possible orientations. This has clear implications for the observed trends in $\Delta S°$ and $\Delta C_p°$. The fact that such subtleties seem to be well accounted for in our highly stable model system indicates that they may indeed be useful for correlating thermodynamic properties such as $\Delta S°$ and $\Delta C_p°$ with concepts such as conformational freedom and hydrophobicity.

The role of Arg methylation in biological systems is just beginning to be determined, and these findings suggest a possible role for enhanced Arg···Trp interactions in the mediation of protein−protein interactions by Arg methylation. Although Arg methylation may not provide a large change in interaction energy with Trp, noncovalent interactions rarely function in isolation. Hence, we expect that a binding pocket which can accommodate a polarized methyl group in DMA via enhanced cation−π interactions, but would not provide hydrogen-bonding sites for an unmethylated Arg, provides the high selectivity necessary for such a subtle post-translational modification to act as a chemical switch for mediating a protein−protein interaction. While our data do not explain the preference, in some cases, for the recognition of asymmetrically methylated arginine over symmetrically methylated arginine, it is clear from Figure 3b that DMAs and DMAa interact with Trp in different geometries, which implies that differently shaped binding sites could easily provide specificity for one form of methylation over the other. We expect that this work will have relevance to the role of Arg methylation in the "histone code".[26]

Finally, another potential extension of this work is to the realm of protein−DNA and protein−RNA interactions. Cation−π interactions have been identified between arginine and adenine and guanine in a number of protein−DNA complexes.[27] Given the number of recent reports of the influence of arginine methylation on RNA transport,[28] the prospect of the enhancement of cation−π interactions upon arginine methylation contributing to protein−DNA or protein−RNA complexes is an intriguing possibility.

Experimental Section

Peptide Synthesis and Purification. 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, 0.07, or 0.05 mmol scale. Non-natural amino acids (DMAa and DMAs) were purchased from Bachem, Inc. and coupled by hand using extended coupling times. Couplings were followed by the Kaiser test.[29] 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. The N-terminus was acylated 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% CH_3CN with 0.1% TFA; B, 95% CH_3CN/5% H_2O with 0.1% TFA). Once purified, peptides were lyophilized to powder and characterized by MALDI 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. 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 second dimension. All spectra were analyzed using standard window functions (sinebell and gaussian). Mixing times of 0.5 and 0.6 s were used in the NOESY spectra. Assignments were made using standard methods as described by Wüthrich.[30] Temperature calibrations were made using methanol and ethylene glycol standards.

Determination of Fraction Folded. To determine the chemical shifts of the fully folded state, 14-residue disulfide-linked analogues of peptides were synthesized with the sequence of Ac-CRWVEVNGOX-ILQC-NH_2, where X = Arg, DMAa, DMAs, K, or KMe3, and characterized by NMR. The disulfide bond between Cys1 and Cys14 constrains the peptide to a β-hairpin. To determine the unfolded chemical shifts, 7-mers were synthesized with sequences Ac-RWVEVNG-NH_2 and Ac-NGOXILQ-NH_2, where X = Arg, DMAa, DMAs, or KMe3. The chemical shifts for residues in the strand and one-turn

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(30) Wüthrich, K. *NMR of Proteins and Nucleic Acids;* John Wiley and Sons: New York, 1986.

residue were obtained from each 7-mer peptide. The fraction folded was determined from eq 10,

$$\text{Fraction Folded} = [\delta_{obs} - \delta_0]/[\delta_{100} - \delta_0] \quad (10)$$

where δ_{obs} is the observed chemical shift, δ_{100} is the chemical shift of the cyclic peptide, and δ_0 is the chemical shift of the unfolded 7-mers. where $T = 298$ K and f is the fraction folded.

$$\Delta G = -RT \ln(f/(1 - f)) \quad (11)$$

Double Mutant Cycles. Single and double mutants of **WDMAs**, **WDMAa**, and **WR** were synthesized by mutating Trp to Val and DMA or Arg to Ser. This resulted in the following peptides, where **X** = R, DMAs, or DMAa: Ac-R**V**VEVNGO**X**ILQ-NH_2, Ac-CR**V**VEVNGO**X**-ILQC-NH_2, Ac-R**W**VEVNGO**S**ILQ-NH_2, and Ac-R**V**VEVNGO**S**ILQ-NH_2. The stability of each mutant hairpin was determined from eqs 10 and 11 as described above, using the following cyclic peptides as controls for the fully folded state: Ac-CR**V**VEVNGO**X**ILQC-NH_2, Ac-CR**W**VEVNGO**S**ILQC-NH_2, and Ac-CR**V**VEVNGO**S**ILQC-NH_2. The magnitude of the side chain−side chain interaction was then determined as shown in Figure 4.

Thermal Denaturations. Thermal denaturations were conducted, following the change in glycine splitting with a 600 MHz Varian NMR spectrometer. Samples were allowed to equilibrate for 7 min at each temperature. Spectra were acquired using 16−32 scans, with 72 000 data points at each temperature. Solvent suppression was achieved using presaturation. Denaturation experiments were run in duplicate, and the temperature was calibrated using methanol and ethylene glycol standards. Thermodynamic data were determined using a nonlinear least-squares fitting algorithm to eqs 1 and 9 using Origin 7.5.[31]

(31) *Origin 7.5*; OriginLab Corp.: Northampton, MA, 2005.

An F test was performed to determine if the improvement in fit from eq 9 was statistically significant.[22] In this case, one cannot rely on comparison of the chi-squared values, since the fit from eq 9 will necessarily be better because it contains two more parameters than eq 1. F is defined as

$$F = {}^1/_2[(N - 3)\chi_1^2/\chi_9^2 - (N - 5)]$$

where N is the number of points in the data set and χ_1^2 and χ_9^2 are the chi-squared values for eqs 1 and 9, respectively. For data sets containing 12 or more data points (the smallest number of points in any of the data sets), an F value of >3.9 indicates that the fit from eq 9 is better than that with eq 1 within 95% confidence limits, and a value of >6.8 indicates $>99\%$ confidence.[22b] For the peptides reported here, only WK gave an F value of less than 3.9, indicating that eq 1 is satisfactory. Of the other peptides, all gave F values at the $>95\%$ confidence level.

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Supporting Information Available: NMR assignments, NOEs, and thermodynamic measurements of reported compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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