The Design of Efficient α-Helical C-Capping Auxiliaries

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Abstract: We have designed a series of C-capping auxiliaries with various chain lengths based on alkyldiamines and monoguanylated diamines. These capping groups were incorporated synthetically at the C-terminus of a derivative of a poly-Ala helix. The free energy of interaction of the capping moiety ($\Delta\Delta G_c°$) with the C-terminus of the helix was assessed by circular dichroism and calculated using a modified Lifson−Roig formalism described by Doig and Baldwin (Doig, A. J.; Baldwin, R. L. *Protein Sci.* **1995**, *4*, 1325) as well as AGADIR2s, an algorithm based on helix−coil transition theory developed by Muñoz and Serrano (Muñoz, V.; Serrano, L. *Biopolymers* **1997**, *41*, 495−509). The alkyldiamine auxiliaries serve as modest helix stabilizers with $\Delta\Delta G_c° = -0.2$ to -0.5 kcal mol^{-1}. The incorporation of monoguanylated diamines at the C-terminus leads to a further increase in α-helicity resulting in $\Delta\Delta G_c° = -0.3$ to -0.7 kcal mol^{-1}. Furthermore, a D-Arg carboxamide auxiliary is demonstrated to be an efficient C-capping residue with $\Delta\Delta G_c° = -1.2$ kcal mol^{-1}. At high concentrations of NaCl (2 M), the capping effect is only partially screened, suggesting that this auxiliary imparts stabilization via charge/dipole interactions as well as through the formation of specific H-bonds between the auxiliary and C-terminal main chain carbonyls.

The α-helix, an essential element in the architectural repertoire of proteins, contains an internal network of hydrogen bonds between the carbonyl of residue *i* and the amide proton of residue $i + 4$.[3] However, this network leaves the helix with four carbonyl groups at the carboxy-terminus and four amide protons at the amino-terminus without hydrogen-bonding partners. In 1988, Presta and Rose as well as Richardson and Richardson predicted that H-bonded interactions between polar side chains and exposed amides at the ends of helices might play a particularly important role in protein folding.[4] These "capping" interactions were hypothesized to serve as motifs which would define the beginning and end of helices as well as provide thermodynamic stability to the intervening helices. Since its inception, this hypothesis has been largely confirmed.[5] In natural proteins, nearly 70% of the N-termini of α-helices engage in capping interactions. Also, variations of the initial N-cap theme have emerged including the "capping box"[6] and the "hydrophobic-staple".[7] These N-capping motifs can stabilize monomeric helices by up to 2 kcal mol^{-1} relative to the unfolded random coil state.[1,6a,7b,8]

By contrast, capping interactions at the carboxy terminus (C-capping) are less frequently observed, occurring in about 30% of the helices in proteins of known structure.[4b] Also, while some canonical C-capping motifs have been observed in the sequences and three-dimensional structures of proteins, they are unable to stabilize the helical structure of short monomeric peptides.[1,9a] For instance, an early study showed that the incorporation of a charged His residue at the C-terminus of a monomeric peptide increases its helical content.[9b] Subsequent quantitative studies indicated that the charged residues His, Lys, and Arg indeed stabilize helix formation when incorporated at a C-cap position, although the free energy of stabilization was only -0.2 and -0.4 kcal mol^{-1} relative to that of Ala.[1] Also, Balaram et al. have recently characterized a Schellman motif present in a heptapeptide.[9c] Structural studies show that, in organic solvents, helical structure is terminated by an achiral residue (aminoisobutyric acid) at the C-terminus which adopts an α_L conformation ($\phi = 50°$, $\Psi = 60°$). However, the extent to which this motif stabilizes helical structure in water is not known. Thus, it appears that C-cap motifs may be more important for helix termination than for stabilization. Therefore, the design of a stabilizing C-capping motif would further our understanding of helix formation, while simultaneously expanding the number of molecular tools available for protein design.

There are at least two possible explanations for the inefficiency of polar side-chain-mediated C-capping interactions: (1) The Cα−Cβ bond of a helical residue does not project directly away from the helical axis but is directed toward the N-terminus at a 52 ° angle. Thus, the short polar side chains of Ser, Asn, and Asp are intrinsically biased toward N-capping rather than C-capping interactions. (2) The longer polar side chains of Arg and Lys, although long enough to "reach back" to H-bond to the C-terminal carbonyl groups, would have to fix many of its side chain torsion angles at an appreciable entropic cost.

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Scheme 1

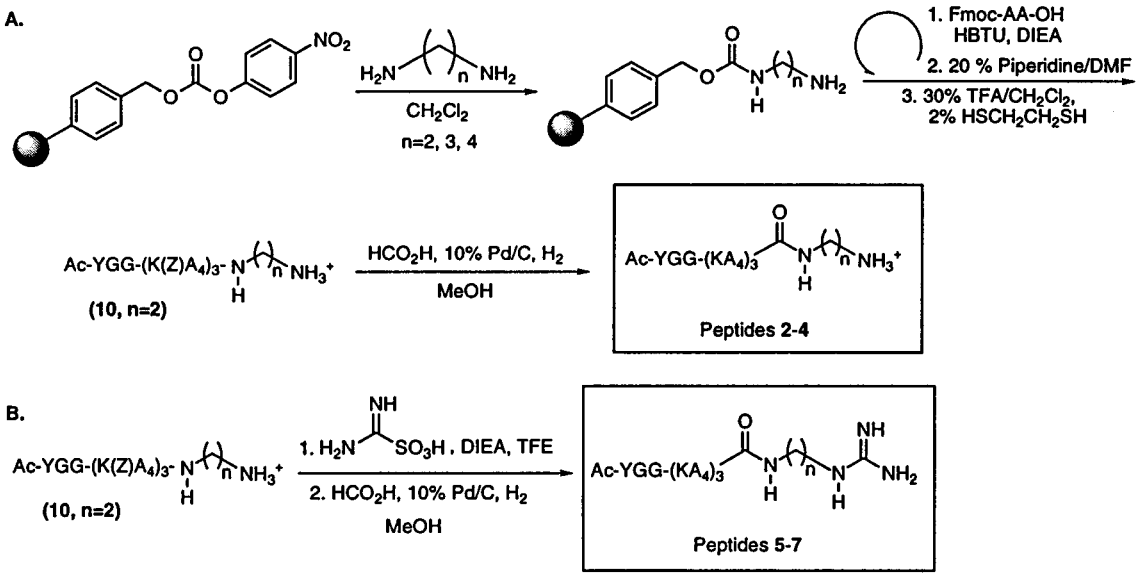

Table 1. Sequence = Ac-Tyr Gly Gly-(Lys Ala$_4$)$_3$-CO-**R**

peptide	C-terminal auxiliary (-**R**)	θ_{222}[a]	$\Delta\Delta G_c°$[b]	$\Delta\Delta G_c°$[c]	$\Delta\Delta G_c°$[d]	$\Delta\Delta G_c°$[e]
1	-NH_2	−15 200				
2	-NH(CH$_2$)$_2$NH$_2$	−17 600	−0.5	−0.5	−0.5	−0.3
3	-NH(CH$_2$)$_3$NH$_2$	−17 700	−0.6	−0.5	−0.5	−0.3
4	-NH(CH$_2$)$_4$NH$_2$	−16 000	−0.2	−0.2	−0.2	0.0
5	-NH(CH$_2$)$_2$NHC(=NH)NH$_2$	−18 400	−0.7	−0.7	−0.6	−0.5
6	-NH(CH$_2$)$_3$NHC(=NH)NH$_2$	−16 700	−0.4	−0.4	−0.4	0.0
7	-NH(CH$_2$)$_4$NHC(=NH)NH$_2$	−18 400	−0.7	−0.7	−0.6	−0.5
8	-D-Ala-$CONH_2$	−15 500	−0.1	−0.1	−0.1	
9	-D-Arg-$CONH_2$	−21 100	−1.3	−1.1	−1.1	−1.1

[a] Mean residue ellipticities (θ_{222} (deg cm^2 dmol^{-1})) were calculated from the CD spectra of 150 μM solutions of peptides (10 mM NaCl, 50 mM acetate buffer, pH 5.0) at 0 °C and are accurate to ±2%.[14a] $\Delta\Delta G_c°$ (kcal mol^{-1}) values were calculated using a Lifson−Roig helix−coil formalism modified to include N- and C-terminal capping effects.[14a] The values of $\Delta\Delta G_c°$ are calculated by comparing an observed θ_{222} value of a peptide to a theoretical θ_{222} of an appropriate helical standard. The latter value was calculated assuming a standard helical length (Nr)[14] of [b]12, [c]15, or [d]18 residues. [e] The $\Delta\Delta G_c°$ values obtained using AGADIR2s[2] were calculated using peptide **8** as the standard of reference since the partition function used by this algorithm is chain length dependent; this ensures that the $\Delta\Delta G_c°$ values obtained do not originate from an increase in chain length. The data were subsequently renormalized to peptide **1** to allow direct comparison with the Lifson−Roig data.

To address these issues, we designed a series of C-capping auxiliaries with various chain lengths based on alkyldiamines and monoguanylated diamines. We envisioned that these extensions would stabilize the neighboring helix by providing H-bond donor(s) to the unsatisfied C-terminal carbonyls, and via electrostatic interactions.

Results and Discussion

Peptide Synthesis. These capping groups were incorporated synthetically (Scheme 1) at the C-terminus of a derivative of a poly-Ala helix originally described by Marqusee and Baldwin[10] resulting in peptides **2**−**7**. Peptides **2**−**7** were prepared starting with 4-nitrophenyl carbonate Wang resin. The resin was reacted with the appropriate alkyldiamine in CH_2Cl_2 for 30 min resulting in alkylamine-charged carbamate Wang resins. These reaction conditions suppress cyclic urea formation, resulting from intramolecular attack of the alkylamino group on the resin-bound urethane (after initial attachment of the diamine). Peptides were prepared on these derivatized resins using standard Fmoc-protocol[12] and benzyloxycarbonyl (Z) side-chain-protected Lys. Cleavage from the resin (30% TFA in CH_2Cl_2 + 2% $HSCH_2$-CH_2SH) afforded Ac-YGG-(K(Z)A$_4$)$_3$-NH(CH$_2$)$_n$NH$_2$, n = 2−4. Side chain deprotection (50% HCO_2H in MeOH + 10% Pd/C) affords peptides **2**−**4**. Peptides **5**−**7** are obtained by guanylation of Ac-YGG-(K(Z)A$_4$)$_3$-NH(CH$_2$)$_n$NH$_2$ (n = 2−4) with aminoiminomethanesulfonic acid[11] and diisopropylethylamine (DIEA) followed by side chain deprotection, as described above. Peptides **1**, **8**, and **9** (Table 1) were synthesized using a standard Fmoc-protocol[12] employing Pal resin.[13] All peptides were purified to homogeneity and characterized by ESI mass spectroscopy.

Assessment of C−Capping Efficiency. The helical content of the resulting peptides were assessed by far UV−circular dichroism (CD) spectroscopy, Table 1. The free energy of interaction of the capping moiety ($\Delta\Delta G_c°$) with the C-terminus of the helix was calculated relative to that observed for a neutral carboxamide (peptide **1**) using a modified Lifson−Roig formalism described by Doig and Baldwin.[14] We have also calculated $\Delta\Delta G_c°$ using the algorithm AGADIR2s, developed by Muñoz and Serrano[2] to demonstrate that our results are general and not merely self-consistent within Doig and Baldwin's formalism. The alkyldiamine auxiliaries serve as modest helix stabilizers (Table 1) with values of $\Delta\Delta G_c°$ only slightly greater than that

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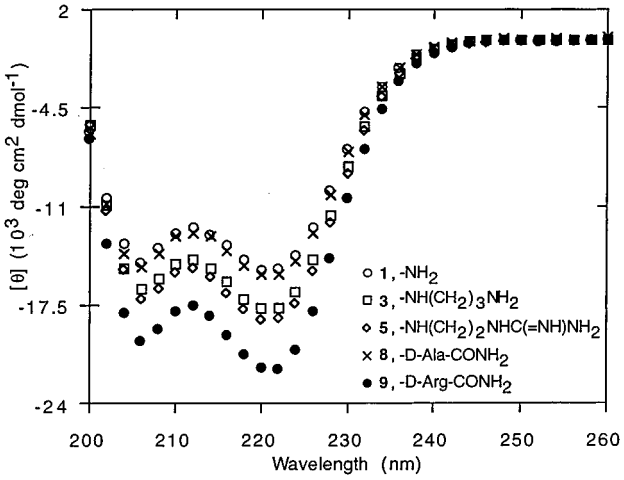

Figure 1. Far UV−CD spectra of 150 μM solutions of peptides **1**, **3**, **5**, **8**, and **9** at 0 °C (pH 5.0, 50 mM acetate, 10 mM NaCl).

expected for the interaction of a formally charged side chain with the partial negative charge at the C-terminus of a α-helix (−0.3 to −0.6 kcal mol^{-1}).[9,15] Indeed, at alkaline pH, where the C-terminal amino group of **2** is deprotonated, the helical content of this peptide decreases.[16]

The incorporation of monoguanylated diamines at the C-terminus leads to a further increase in α-helicity (Table 1), possibly arising from additional H-bonding interactions not possible in the simple alkyldiamine auxiliaries. The ability of the alkyldiamine and monoguanylated diamine extensions to stabilize α-helical structure shows a strikingly different length dependence, (Table 1). An alkyl chain length of three methylenes is optimal for the alkyldiamine extensions, whereas a chain length of either two or four methylenes is optimal for the guanido-based extensions (Figure 1; Table 1).

We next explored the addition of H-bonding groups to peptide **7** to further stabilize the C-capping interaction. In particular, the addition of a carboxamide to the C_1 methylene of the auxiliary should enhance the capping interaction by providing an additional H-bond donor to exposed C-terminal acceptors as well as reducing the number of energetically accessible rotamers of the alkyl portion. Thus, this modification should decrease the entropic requirements associated with forming the capping interaction. The carboxamide was added at the prochiral C_1 position yielding a D-carboxamide derivative of Arg. For the reasons discussed above, L-Arg is known to be a modest C-capping residue ($\Delta\Delta G_c^\circ = -0.3$ kcal mol^{-1}).[1] By contrast, D-Arg carboxamide leads to a dramatic increase in helical content (Figure 1), corresponding to a $\Delta\Delta G_c^\circ$ of −1.1 to −1.3 kcal mol^{-1} (Table 1).

The electrostatic contribution to this interaction was probed by comparing the salt dependence of the helical content of peptides **8** and **9** between 0.01 and 2.0 M NaCl (Table 2). The helical content of **8**, which terminates with D-Ala carboxamide, increases with increasing ionic stength ($\theta_{222} = -15\,500$ and −18 400 deg cm^2 dmol^{-1} at 0.01 and 2 M NaCl concentrations, respectively), possibly due to a decrease in the electrostatic repulsion between the Lys side chains. By contrast, the value of θ_{222} for **9**, which terminates with D-Arg carboxamide, was practically invariant in this range of ionic strength. This finding suggests that the unfavorable electrostatic interactions between the Lys side chains in the α-helical conformation of **9** are as easily screened by exogenous electrolyte as the favorable electrostatic interactions between the C-terminal guanidinium group and the peptide carbonyls.

Table 2. Sequence = Ac-Tyr Gly Gly-(Lys Ala_4)$_3$-CO-**R**

peptide	C-terminal auxiliary (-**R**)	NaCl (M)	θ_{222}[a]	$\Delta\Delta G_c^\circ$ (**9** → **8**)[b]
8	-D-Ala-$CONH_2$	0.01	−15 500	
		0.5	−17 800	
		1.0	−18 700	
		2.0	−18 400	
9	-D-Arg-$CONH_2$	0.01	−21 100	−1.0
		0.5	−21 100	−0.5
		1.0	−21 500	−0.4
		2.0	−20 900	−0.4

[a] Mean residue ellipticities (θ_{222} (deg cm^2 dmol^{-1})) were calculated from the CD spectra of 150 μM solutions of peptides (50 mM acetate buffer, pH 5.0) at 0 °C and are accurate to ±2%.[14a] [b] $\Delta\Delta G_c^\circ$ (**9** → **8**) is the difference in $\Delta\Delta G_c^\circ$ for peptide **9** vs. **8** at the indicated salt concentration calculated using the modified Lifson−Roig formalism assuming a standard helical length (Nr)[14] of 15.

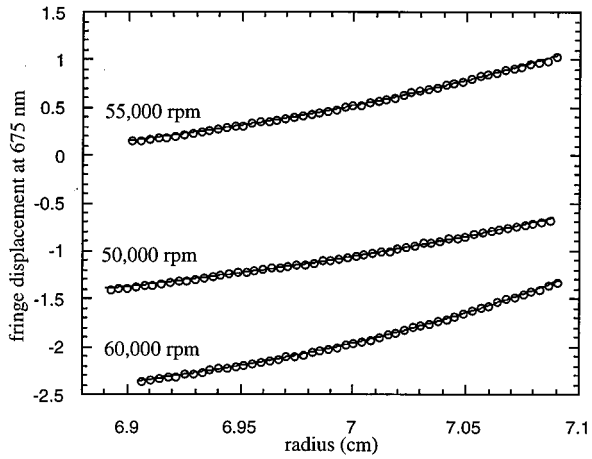

Figure 2. Sedimentation equilibrium ultracentrifugation analysis of peptide **9** demonstrates that this peptide is monomeric ($MW_{expected}$ = 1729 and MW_{fit} = 1770).

The difference in helical content between peptides **8** and **9** decreases with increasing ionic strength and levels off near 1.0 M NaCl. Table 2 compares $\Delta\Delta G_c^\circ$ (**9** → **8**) (the difference in $\Delta\Delta G_c^\circ$ for **8** and **9**) at various salt concentrations. The value of $\Delta\Delta G_c^\circ$ (**9** → **8**) levels off at salt concentrations greater than 1 M, reaching a constant value of −0.4 kcal mol^{-1}. This suggests that the D-Arg carboxamide auxiliary stabilizes the helix through the formation of specific H-bonds as well as through more easily screened charge/dipole interactions.[17]

Also, when D-Arg carboxamide is replaced with D-Ala carboxamide the helix stabilizing effect is completely lost, indicating that the carboxamide portion of the D-Arg auxiliary alone is not solely responsible for the stabilization of the helix. Sedimentation equilibrium ultracentrifugation (Figure 2) demonstrated that peptide **9** is monomeric indicating that the observed enhanced helicity was a result of intramolecular

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(16) Peptides **1** and **2** show opposite pH-dependent behavior. The magnitude of θ_{222} for a 150 μM solution of peptide **1** increases from −15 200 to −19 900 deg cm^2 dmol^{-1} as the pH is increased from 5.0 (50 mM acetate, 10 mM NaCl) to pH 10.0 (50 mM CAPS, 10 mM NaCl). This increase in α-helical content arises from decreased electrostatic repulsion of the Lys side chains in the helical state; the corresponding values for a 150 μM solution of peptide **2** at pH 5.0 and 10.0 were −17 600 and −14 300 deg cm^2 dmol^{-1}, respectively, indicative of a decrease in α-helical content. Thus, the ethylenediamine auxiliary promotes helix formation in its charged state but is detrimental in its neutral state.

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interactions and not due to the oligomerization of individual helices. Preliminary molecular dynamics studies suggest that stability is imparted to the helix via the formation of bidentate H-bonds between the guanido portion of the auxiliary and exposed C-terminal carbonyl groups, and NMR spectroscopic studies are in progress to confirm this mode of interaction.

Conclusion

These studies provide the first systematic exploration of modified alkyldiamines as α-helical C-capping residues. The synthetic methodology presented may be easily modified to allow the incorporation of a large array of conformationally restricted diamines which may provide greater enhanced helical stability. Further, D-Arg may be conveniently incorporated at any position within a peptide or protein sequence, providing a useful tool for protein design.

Experimental Section

General Methods and Material. The 4-nitrophenyl carbonate Wang and Pal resins were purchased from Calbiochem-Novabiochem International, Inc. Methylene chloride (CH_2Cl_2), dimethylformamide (DMF), piperidine, methanol (MeOH), trifluoroethanol (TFE), diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), ethanedithiol (HS-$(CH_2)_2$SH), formic acid (HCO_2H), 10% Pd/C, ethylenediamine, 1,3-diaminopropane, and 1,4-diaminobutane were purchased from Aldrich Chemical Co. and used without further purification. The appropriate side-protected Fmoc-amino acids and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from either Advanced Chemtech or Calbiochem-Novabiochem and were used without further purification. Aminoiminomethanesulfonic acid was prepared by the peracetic acid oxidation of formamidinesulfinic acid as described by Mosher.[11] Peptides were purified using either a preparative Vydac C4 or C18 peptide/protein column. Solvent A was composed of water and 0.1% TFA, and solvent B was composed of 90% acetonitrile, 10% water, and 0.1% TFA.

Synthesis of Peptides. Peptides **1**, **8**, and **9** were prepared on Pal resin using a standard Fmoc protocol.[12] Peptides **2**−**4** were prepared according to identical protocols, which will be described in detail for peptide **2**. Peptides **5**−**7** were also prepared according to a general procedure, which will be described by the synthesis of peptide **5**.

Synthesis of Peptide 2 (Ac-YGG-$(KA_4)_3$-NH$(CH_2)_2NH_2$). A manual solid-phase reaction vessel was charged with 4-nitrophenyl carbonate Wang resin (0.75 g, 0.5 mmol, loading = 0.67 mmol g^{-1} resin) and enough CH_2Cl_2 to swell the resin (2−3 mL). Ethylenediamine (0.33 mL, 5 mmol) was added via syringe, and the reaction was shaken for 30 min (venting of the reaction vessel is necessary for the first 10 min due to the gerenation of excessive pressure). After 30 min, the resin was drained and washed with CH_2Cl_2 (3 × 1 min), DMF (3 × 1min), and CH_2Cl_2 (3 × 1min). Ethylenediamine (0.33 mL, 5 mmol) was recoupled to the resin overnight to ensure maximum loading after which time the resin was washed as desribed above. Standard Fmoc protocol[12] employing HBTU activation was used to generate the resin-bound benzyloxycarbonyl (Z) side-chain-protected peptide Ac-YGG-$(K(Z)A_4)_3$-NH$(CH_2)_2$NH resin. Subsequent peptide cleavage from the resin was accomplished by suspending the resin-bound peptide in 5 mL of 30% TFA/2% ethanedithiol in CH_2Cl_2 under N_2 atmosphere for 2 h. This mixture was then filtered, and 20 mL of 2-propanol was added to the filtrant which was subsequently evaporated under reduced pressure to near dryness. Cold diethyl ether was added to precipatate crude **10** which was collected by filtration and dried under high vacuum. Deprotection of the Z-protected Lys residues of **10** was performed as follows: a 50 mL round-bottomed flask was charged with crude **10** (50 mg, 23.5 μmol), 5 mL of MeOH, and 7 mL of 98% formic acid. A suspension of 10% Pd/C (20 mg) in 2 mL of MeOH was then added via pipet, and the reaction was stirred under 1 atm of H_2 for 2 h. The reaction was filtered, and the residual Pd/C was washed with MeOH (3 × 5 mL). The filtrant was evaporated to dryness, and the resulting residue was dissolved in 0.1% TFA in H_2O and lyophilized yielding crude Ac-YGG-$(KA_4)_3$-NH$(CH_2)_2NH_2$ **(2)** as a biege solid. A small sample of crude **2** (2 mg) was purified for CD analysis by preparative RP-HPLC employing a linear gradient from 7% to 37% solvent B over 60 min. MS (ESI): 1616.5 [$(M + H)^+$, calcd 1616.9]. MS for peptide **3**: 1630.8 [$(M + H)^+$, calcd 1630.9]. MS for peptide **4**: 1644.9 [$(M + H)^+$, calcd 1645.0].

Synthesis of Peptide 5 (Ac-YGG-$(KA_4)_3$-NH$(CH_2)_2$NHC(═NH)-NH_2). A 2 mL round-bottomed flask was charged with crude **10** (20 mg, 9.38 μmol) and 0.3 mL of TFE. The solution was stirred, and DIEA (13 μL, 75 μmol) was added via syringe followed by aminoiminomethanesulfonic acid (9.2 mg, 75 μmol) as a solid. The reaction was stirred for 3 h, at which time ethylenediamine (5 μL, 75 μmol) was added via syringe to quench the reaction followed by MeOH to a final volume of 5 mL. Ac-YGG-$(K(Z)A_4)_3$-NH$(CH_2)_2$NHC(═NH)-NH_2 **(11)** was immediatley purified by injecting the final solution onto a preparative C4 column employing isocratic conditions of 0% B for 10 min followed by a linear gradient of 0−100% B over 100 min yielding a white powder after lyophilization. Deprotection of the Z-protected Lys side chains of **11** was accomplished as described above affording pure **5** after a small sample (2 mg) was purified for CD analysis (preparative C18 employing a linear gradient of 7% to 37% solvent B over 60 min). MS (ESI): 1659.0 [$(M + H)^+$, calcd 1658.9]. MS for peptide **6**: 1673.0 [$(M + H)^+$, calcd 1673.0]. MS for peptide **7**: 1688.0 [$(M + H)^+$, calcd 1687.0]. MS for peptides **1**, **8**, and **9**: peptide **1**, 1574.3 [$(M + H)^+$, calcd 1573.9]; peptide **8**, 1645.0 [$(M + H)^+$, calcd 1644.9]; peptide **9**, 1730.0 [$(M + H)^+$, calcd 1730.0].

Circular Dichroism Studies. CD spectra were collected on an AVIV 62DS spectropolarimeter using a 1 mm quartz cell. Peptide samples were prepared from stock solutions in water and diluted to 150 μM with desired buffer. The concentration of peptide solutions were determined by Tyr absorbance at 276 nm ($\epsilon = 1450$ cm^{-1} M^{-1}). Mean residue ellipticity (θ_{222}) was calculated using the equation $(\theta_{222}) = (\theta_{obsd}/10lc)/r$, where θ_{obsd} is the ellipticity measured at 222 nm in millidegrees, l is the length of the cell (cm), c is the concentration (M), and r is the number of residues. Fraction helix (f_H) was calculated according to the Luo and Baldwin[14b] equation f_H (0 °C) = $(\theta_{222} - 2220)/(-44000(1 - 3/Nr) - 2220)$, where Nr is the number of residues in a helical conformation. A sensitivity analysis in which the value of θ_{222} for 100% helix formation was varied by ±15% causes small systematic changes in the free energy of helix formation for the individual peptides but practically invariant changes in $\Delta\Delta G_c°$. Initiation (v), propagation (w), n-capping (n), and C-capping (c) parameters used for the calculation of $\Delta\Delta G_c°$ can be found in the Supporting Information.

Sedimentation Equilibrium Ultracentrifugation. Sedimentation equilibrium data were collected at 2 °C for a 150 μM sample of peptide **9** (50 mM acetate, 10 mM NaCl, pH 5.0) on a Beckman XL-I Analytical ultracentrifuge equipped with interference optics. Peptide concentration was determined by tyrosine absorbance ($\epsilon_{275nm} = 1450$ M^{-1} cm^{-1}). Data were collected at three speeds (50 000, 55 000, and 60 000 rpm) and fit globally as previously described[18] using the software package Igor Pro (WaveMetrics, Inc.).

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Supporting Information Available: Circular dichroism (CD) spectra of peptides **1**−**9**, pH-dependent CD spectra of peptide **2**, NaCl-dependent CD spectra of peptides **8** and **9**, and tables of fractional helical content for peptides **1**−**9** as a function of helix length, the modified Lifson−Roig C-capping paramters (c), and the corresponding free energies of helix formation for peptides **1**−**9** (5 pages). See any current masthead page for ordering information and Web access instructions.

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