Optimal N-Caps for N-Terminal Helical Templates: Effects of Changes in H-Bonding Efficiency and Charge

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Received March 28, 2001

Abstract: A family of efficient helix-initiating N-terminal caps X-Hel is introduced that expand the scope and versatility of the previously reported reporting conformational template Ac-Hel, (Kemp, D. S.; Allen, T. J.; Oslick, S. *J. Am. Chem. Soc.* **1995**, *117*, 6641−6657) and a working principle for predicting cap performance is described, based on structurally specific intramolecular hydrogen bond formation. Replacement of the *N*-acetyl by urethane, urea, or sulfonamide generated less efficient polypeptide helix inducers. The *N*-formyl cap is found to be equivalent to the *N*-acetyl and may provide more convenient quantitative helix reporting properties. Anionic N-caps derived from the series X = $^{-}O_2C$-$(CH_2)_n$-CO, $0 \leq n \leq 3$, are superior to *N*-acetyl, as are *N*-acylglycyl and *N*-acyl-β-aspartyl. The latter pair of caps permit introduction of the X-Hel functionality within a polypeptide chain, allowing control of helicity of a peptide sub-sequence. Applications of these capping functions are discussed. This work has been focused primarily on immediate practical goals directed toward enhancing the maximum helicity of isolated short to medium-sized peptides in aqueous solution, but its developing concepts and working hypotheses are likely to significantly enhance our understanding at a chemical level of the protein folding problem.

The N-terminal peptide capping residue Ac-Hel, Figure 1, a conformationally locked derivative of the dipeptide Ac-Pro-Pro, is a versatile tool for initiating, enhancing, and quantitating polypeptide helicity.[1] Previous work has established that the acetamide function of Ac-Hel, which can be viewed as a cap within a cap, is a modular component that controls the helix-enhancing properties of Hel itself. Can one improve on the helix-inducing capacity of the acetamide component of the Ac-Hel cap? We have modified our published synthesis[2] of the Hel function to generate analogues X-Hel, in which the acetyl of Ac-Hel is replaced by other acyl or hydrogen bond donor functions. In this report we provide details of these new syntheses and evaluate the properties of a new generation of N-terminal helix initiation templates.

Peptide helicity is dramatically enhanced by a variety of N- and C-caps, yet an understanding of the mechanisms of capping stabilization is incomplete, and the upper limit on cap-induced helicity remains largely unexplored. This report outlines our recent efforts to address these issues. Relying on the X-Hel framework we have constructed a new database of capping X-functions and their experimental helix-inducing efficiencies. Along with new nonamide H-bonding caps, this data base contains X-caps that have been previously reported to increase helicity when linked N-terminally to simple peptides. In building this data base, we have postulated that many of the efficient helix stabilization mechanisms that allow an X-cap to induce strong helicity in a simple peptide will also operate to increase the helix-inducing efficiency of the corresponding X-Hel cap.

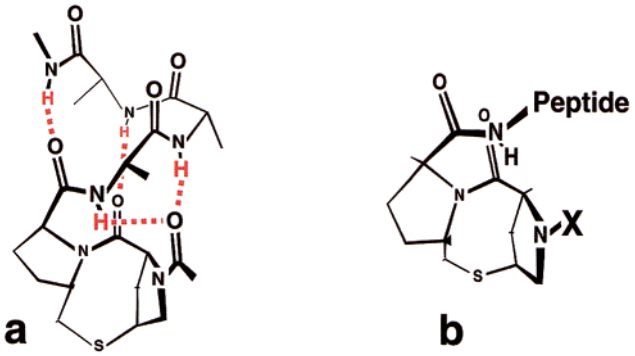

Figure 1. The structures of the helix inducing conformational templates Ac-Hel and X-Hel. (a) A tripeptide N-terminus bonded to the N-cap Ac-Hel. Intramolecular hydrogen bonds form between four NH donor functions and three C═O acceptors introduced by Ac-Hel. (b) The generic Hel-peptide structure, in which H-bond acceptor functions X replace the acetamide of Ac-Hel.

Three practical goals have driven this study. We seek X-Hel N-caps that share the novel helix reporting feature of Ac-Hel. We also seek X-Hel caps that are substantially better than Ac-Hel at initiating and enhancing peptide helicity. This goal has particular urgency, given the current need for CD calibration standards in the form of short to medium-sized peptides of defined length that approach 100% helicity.[3] We finally seek an X-Hel function that strongly stabilizes a helix within peptide residues that are C-terminal to it, that can be incorporated within a peptide sequence, and that blocks N-terminal helix extension. This function could serve as an N-terminal equivalent of β-aminoalanine, which we have recently demonstrated to be a strong C-terminal helix boundary definer.[4] Used jointly, this

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10.1021/ja010812a CCC: $20.00
Published on Web 09/26/2001

pair could permit construction of designer proteins[5] containing helices that are confined to particular regions within the peptide sequence. In this report we demonstrate examples of X-Hel functions that meet two of these three goals.

Background and Design Plan

The large helix stabilizing effects reported for simple peptide N-caps set a high standard for our goal of optimizing X-Hel helix stabilizers. They also provide clues to the mechanisms of cap stabilization. In 1987 Shoemaker et al. noted helix stabilization for succinyl capped analogues of the C-peptide of RNAse A, which they attributed to an interaction between the backbone amide helix dipole and the negative charge of the N-terminal succinate.[6] Drawing from the protein X-ray crystallographic database, in 1988 Presta and Rose noted sequence correlations within the end regions of helices found in native structures of globular proteins and argued that helix start and stop signals are likely to exert more control over peptide helicity than stabilizing effects attributable to helix propagation.[7] In 1994, using a 15 residue helix-prone model peptide, Forood et al. noted that replacement of its N-terminal acetyl by a negatively charged succinyl cap doubled the value of $-[\theta]_{222}$, implying an equivalent increase in fractional helicity.[8] They also noted little change in $-[\theta]_{222}$ when the carboxylate anion of the N-succinyl was replaced by a sulfonate anion or by an uncharged methyl sulfone. All these capped peptides are exceptionally helical. Studies by Nambiar et al.[9] and by Doig and Baldwin[10] with peptides containing 12 to 17 residues further showed that, relative to an uncharged alanine residue, the *N*-acetyl cap increases $-[\theta]_{222}$ by an average of 70%. For the entire peptide series the span of increase resulting from *N*-acetylation was 40−120%, with larger increases observed for shorter peptides or for weakly helical peptide sequences. Strongly helix stabilizing N-capping effects were also seen for natural amino acids normally classified as helix breakers; for example, a 40 to 60% increase of $-[\theta]_{222}$ was observed when N-terminal unprotonated alanine residues were replaced by asparagine, glycine, or serine residues, or by negatively charged aspartates.

Three significant generalizations follow from these studies. Large increases in peptide helicity can result from N-capping, even by functions as simple as an acetyl. Negatively charged N-caps are very strong helix stabilizers. Uncharged caps containing a variety of polar functions can match the effectiveness of negatively charged N-caps.

These generalizations suggest key features of mechanisms by which highly efficient N-caps may stabilize helices. At an N-terminus of an α-helix formed by an uncapped peptide, the NH functions of the first four peptide amide residues lack intramolecular H-bonds. As represented in Figure 2a, N-capping by a simple acyl-derived H-bond donor such as an acetamide

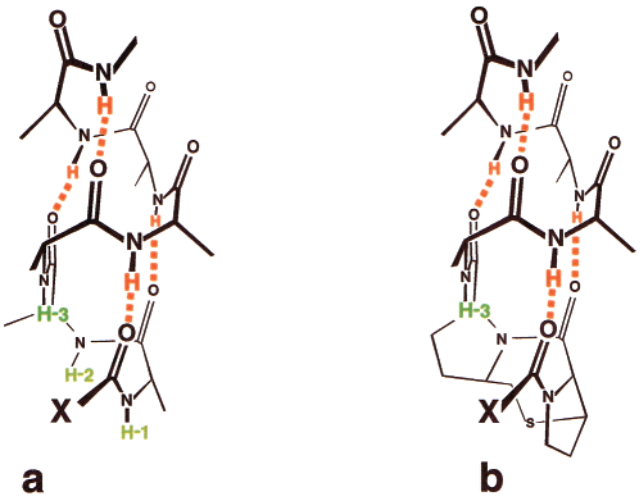

Figure 2. Comparison of templated and nontemplated N-capped peptides. (a) The N-terminus of an *N*-acyl capped α-helical peptide presents three NH functions that lack H-bonding capacity within the helix (labeled H-1, H-2, and H-3). (b) The corresponding N-terminus of an *N*-acyl-Hel peptide, in which the constrained Pro-Pro sequence of Hel corresponds to the first two peptide residues of **2a**. The potential single H-bonding donor site H-3 is labeled.

can generate an H-bond with a single NH, but the remaining three NH residues must find H-bond donors within a complex of hydrating water molecules. The additional stabilization noted by Forood et al. when the monovalent H-bonding acetyl N-cap is replaced with a potentially bivalent succinyl can be explained if its charged carboxylate stabilizes the N-terminus by assuming the helix stabilizing roles of one or more water molecules. Binding of anions by a helix N-terminus, usually attributed to charge-dipole interactions, is well documented by protein X-ray crystallographic data.[11] However, the nearly equivalent helix stabilizing effects of uncharged and negatively charged bivalent caps reported for N-capped peptides by Forood et al. suggest that the helix-stabilizing capacity of the second polar functions of their N-caps may correlate better with H-bonding acceptor affinity.

In certain respects the picture of Figure 2a is simplistic, but a more incisive view of the roles of N-caps requires a more detailed and rigorous model for the structure of the highly functionalized and solvated N-terminus of a normal helical peptide. Such models are unlikely to be forthcoming, given the limitations of currently available molecular modeling protocols and the degree to which conformational averaging complicates interpretation of CD-derived peptide helicity. To design a new generation of N-caps one would like answers to a series of simple questions. Does a strongly helix stabilizing polar or charged H-bond acceptor that is part of a peptide N-cap act by interacting directly with a specific backbone NH residue of the peptide? Does it interact directly with more than one NH via bifurcated H-bonds? Does it stabilize the helix indirectly by local charge-dipole effects or by modifying the structure or affinities of the water molecules within the N-capping region? These questions cannot be answered rigorously, but as a working model for design of new experiments, we have used heuristic comparisons of models for the structures of normal N-capped helical peptides with models for analogous N-capped Hel-peptide conjugates. This comparison leads us to focus attention on H-bond formation by a particular NH of the peptide sequence.

The structure of the interface between a peptide and its Ac-Hel cap can be viewed in two ways. The Ac-Hel cap can be

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viewed as a unit, or as a peptide-derived module containing an internal N-cap. Viewed as a unit (Figure 1), the Ac-Hel cap provides three amide carbonyl oxygens as H-bonding acceptor sites for the four N-terminal NH functions of the peptide. Not surprisingly, the Ac-Hel cap is found experimentally to be 5 to 10 times more efficient than the simple acetyl N-cap, which provides only one donor site.

Alternatively, one can regard an X-Hel function as a module that provides a rigid, helically oriented diproline that extends the length of the peptide sequence N-terminally by two amino acid residues. Its X-function then caps this extended sequence by introducing an N-terminal H-bonding acceptor site. One can now compare helix stabilizing roles of X-caps for the simple peptide and for its lengthened Hel-peptide analogue.

Specifically this comparison shows that for the Hel analogue, Figure 2b, two of the secondary amide NH functions belonging to the simple X-capped peptide of Figure 2a have been replaced by proline-type tertiary amides which lack H-bonding donor capacity. Four potential H-bonding donor sites appear at the N-terminus of a normal α-helix, but in the X-Hel analogue only one remains with an unsatisfied H-bonding valence. As seen in Figure 2b, this site belongs to a residue that corresponds to residue 3 of the simple peptide sequence of Figure 2a.

This structural comparison suggests an experimental comparison of the $-[\theta]_{222}$ changes that result for two Ac→X substitutions: the first for the simple N-Ac-peptide, and the second for an analogous Ac-Hel-peptide. For the simple peptide, a large increase in $-[\theta]_{222}$ is known to occur if the Ac cap, which carries only a single H-bonding acceptor site, is replaced by a strongly stabilizing cap X, such as succinyl with its two potential H-bonding acceptor sites. For the Ac-Hel peptide, if a correspondingly large increase in $-[\theta]_{222}$ is seen if Ac is replaced by the same X, then the magnitude of the stabilizing X interactions that occur within the simple peptide must be similar in magnitude to those that occur within the X-Hel peptide. Since only the H-bonding interaction at peptide site 3 appears in the latter case, this interaction must also largely control X-derived helicity for the simple peptide. Provided the effects of the two Ac→X mutations are similar, formation of the H-bond at site 3 must be a necessary and sufficient condition for strong helix stabilization.

One can argue independently for the control of peptide helicity by H-bonding at peptide site 3. Modeling of the N-terminal region shown in Figure 2a suggests that with any of the known strongly helix stabilizing bivalent N-caps, only the NH of residue 3 can form an intramolecular H-bond that defines a loop with helical structure. The NH of residue 3 thus may play a unique role in N-terminal helix initiation.

In this study we construct a new X-Hel library by studying a range of simple and bivalent X-caps. We address four experimentally resolvable questions. First, for X-Hel-peptide analogues, how large a change in helix stabilization occurs if the acetamide H-bonding site of Ac-Hel is replaced by a variety of good hydrogen bond acceptors that are not amides? Second, among simple acyl functions is the acetyl maximally efficient as a helix stabilizer? Third, are members of the X-Hel family for which X is glycyl equivalent to Ac-Hel in helical N-capping efficiency and does replacement by succinyl to form succ-Hel increase N-capping efficiency? In other words, does an Ac→X helicity change that is seen for simple peptides parallel the corresponding change seen for Hel-peptide conjugates? Resolution of these questions should also address two goals: optimization of the helix-inducing potential of the X-Hel family and clarification of the mechanisms by which the helix-stabilizing

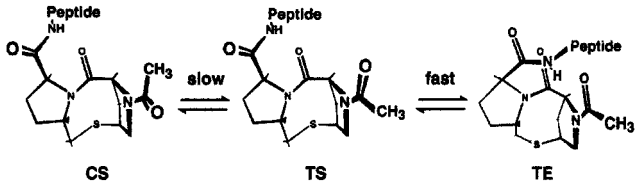

Figure 3. The three conformational substates of Ac-Hel peptide conjugates, as identified by analysis of their ^{1}H NMR spectra.[1]

effects of X operate. Both issues are pertinent to our overall practical objective of increasing the efficacy and versatility of the Hel family of helix-inducing peptide N-caps.

Alternatives to Amide Functions at the X-Hel Junction

In this section we report results of replacing the Ac-derived amide group of Ac-Hel by the pivalamide group $(CH_3)_3C$-CO-N, by a urea group $(CH_3)_2N$-CO-N, a urethane CH_3-O-CO-N, and a sulfonamide CH_3SO_2-N. The likely effectiveness of these substitutions is suggested by literature data. If proton basicities largely determine the relative efficacies of these helix-inducing N-caps, then the helicity order for X-Hel peptide conjugates is expected to be urea ≥ amide > urethane > sulfonamide,[12] and a similar order is predicted for the first three series members from the limited data available for the strength of the intramolecular H-bond of peptide β-turns.[13] However, if the data observed by Forood et al. for simple peptides correlates with that of X-Hel derivatives, then a sulfonamide is expected to be more helix stabilizing than an amide. We included the highly hindered X = *N*-pivaloyl derivative in this study of nonamide caps because of an early report that short peptides capped by *N*-pivaloyl-proline exhibit unusually high helicity.[14]

The helix-initiating efficiency and the unique helix reporting feature of the Ac-Hel cap are controlled by the relative energetics and rates of equilibration of its conformational substates. These in turn are strongly influenced by the properties of its acetamido internal cap. The replacement of Ac-Hel by X-Hel thus is expected to modify helix-inducing efficiency directly by changing the H-bonding affinity of a major acceptor site and indirectly by modifying the relative stabilities of helical and nonhelical Hel-derived conformational states.

As noted in Figure 3, modeling and NMR data establish the presence of three distinguishable conformational states for the Ac-Hel function, cs, ts, and te.[1] Only the te state is capable of initiating helical structure in a helically disposed N-terminally linked peptide. Since ts and te are in rapid equilibrium at ambient temperatures on the NMR time scale, the three states can profitably be regrouped as c = cs and t = ts + te. The c and t states orient the acetamido function in respective s-cis and s-trans rotamers at the CO−N bond, and at ambient temperatures these are in slow equilibrium on the NMR time scale, leading to separate ^{1}H NMR c and t state resonances. Ratios of helical to nonhelical mole fractions of Ac-Hel-peptide derivatives have been shown to be proportional to t/c, the relative integrated intensities of these resonances.

The barriers to rotation about N−CO bonds of ureas and RSO_2−N bonds of sulfonamides are expected to be substantially lower than those of amides.[15] The NMR resonances at ambient temperatures for the corresponding peptide X-Hel conjugates are thus expected to comprise a single abundance-weighted

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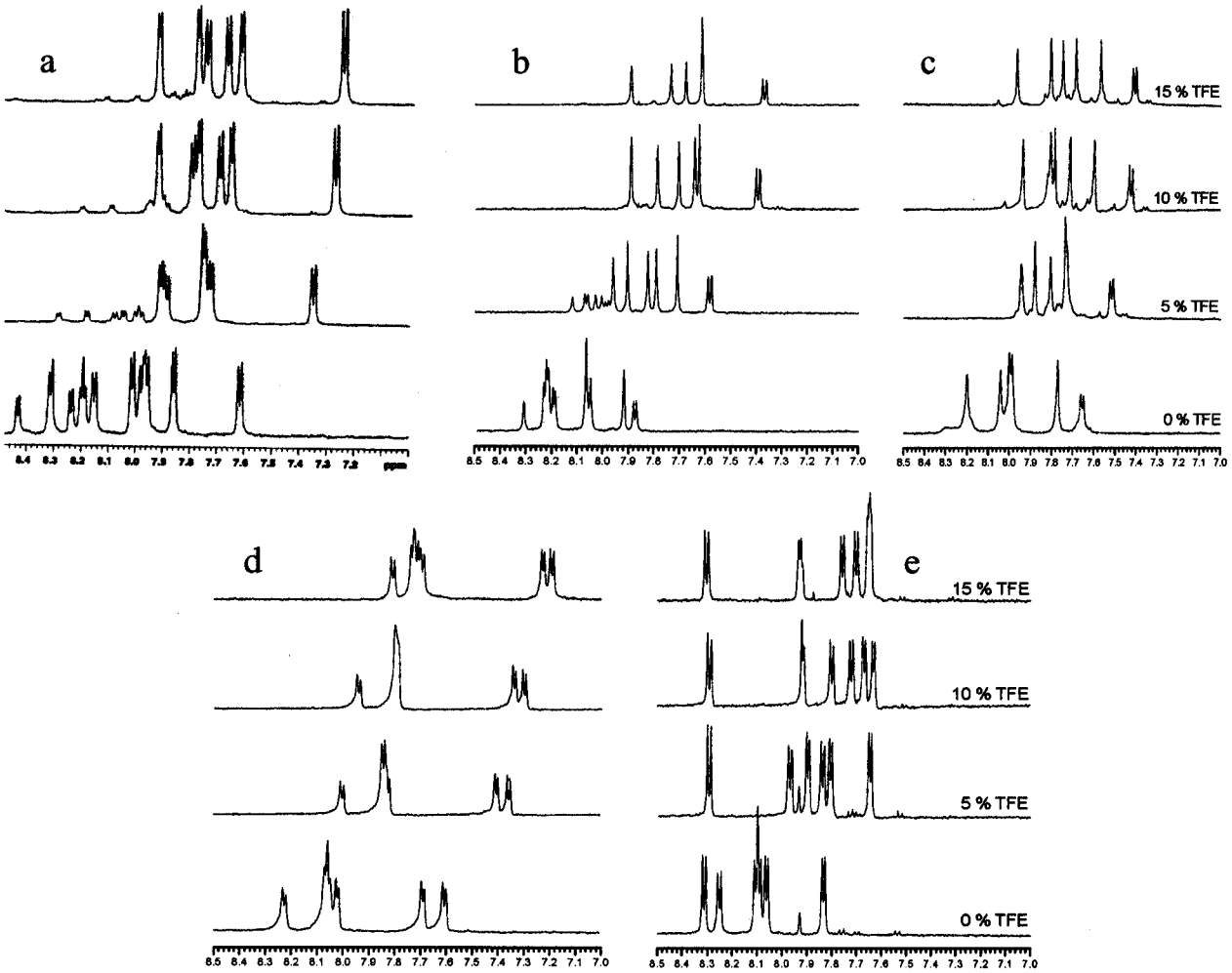

Figure 4. NH regions in ^{1}H NMR spectra of (a) AcHel-A_6-OH (**1a**), (b) MocHel-A_6-OH (**1b**), (c) PivHel-A_6-OH (**1c**), (d) MesHel-A_6-OH (**1d**), and (e) DMUHel-A_6-OH (**1e**) in water and water/TFE−mixtures (mol % TFE given) at 25 °C. Chemical shifts in ppm, referenced to TSP (trimethylsilyl propionate).

conformational average of resonances expected for rotamers at the X-CO bond. Peptide conjugates of these X-Hel caps are thus predicted to lack convenient NMR-derived helicity-reporting features.

Although nearly optimal as a helicity reporter, Ac-Hel is sub-optimal as a helix initiator.[1] The presence of significant amounts of nonproductive cs and ts conformers weakens it as a helix-stabilizing N-cap. Moreover, modeling and NMR evidence suggest that its acetamido cap is intrinsically deficient as an N-terminal H-bond acceptor.[1,16] As seen in Figure 2b, the N-terminus of an α-helical peptide contains four backbone NH H-bond donors, but the Ac-Hel cap provides only three amide carbonyl oxygen H-bond acceptors. As seen in Figure 1, modeling predicts that the carbonyl oxygen of the acetamide function takes part in bifurcated hybrid 3_{10}-α-type H-bond formation to the NH functions of residues 1 and 2, with a substantial out-of-plane distortion for the first of these H-bonds. For Ac-Hel-Ala_n-OR, R = H or *t*-Bu, in a variety of solvents including water and trifluoroethanol−water, relative to the other ^{1}H NMR t-state NH resonances, that of NH-1 shows an anomalously large upfield shift with an increase in the peptide length *n* or in the mole fraction of TFE, χ_{TFE}. This shift is consistent with the modeling prediction of a helicity-correlated change in the H-bonding environment at this site, attributable to structural adjustments in the sub-optimal fit between the H-bonding NH donor functions of the first two amino acids and the acceptor function of the Ac-Hel acetamide. Absence of NMR evidence for length and TFE-dependent structural adjustments provides a criterion for judging candidates for replacing Ac-Hel.

The dependencies of δ on χ_{TFE} in water for the peptide NH resonances of Ac-Hel-Ala_6-OH and the four new X-Hel-Ala_6-OH derivatives of this study are shown in Figure 4a−e. In water the δ NH values for alanine residues in unstructured states have been reported to fall in the range of δ 8.15−8.45 ppm, while those of tightly structured helicies fall in the range of δ 7.5−8.1 ppm.[17] As seen in Figure 4a, in water the NH region of the ^{1}H NMR spectrum of Ac-Hel-Ala_6-OH contains twelve partially overlapping resonances; these correspond to the resonances of the six t and six c states. The δ values for the c state resonances fall in the predicted unstructured range while the t state resonances, which correspond to a frayed te state helix with approximately equal contribution from an unstructured ts state, fall within the helical range. Adding TFE in increasing amounts has three effects.[1,18] First, the c state resonances are dramatically diminished in relative intensity for $\chi_{TFE} \geq 0.05$ and the t state

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resonances dominate the spectra. Second, with the exception of the resonance of NH-1, the t state resonances are consolidated in the narrow region $7.75 < \delta < 7.95$ ppm. Third, the t state resonance for NH-1 shifts from δ 7.62 to 7.25 ppm.

Inspection of the ^{1}H NMR spectra in water of the NH regions for four X-Hel-Ala$_6$-OH derivatives shown in Figure 4b−e shows that only the urethane spectrum shown in Figure 4b contains more than six detectable NH resonances. This spectrum in water alone is consistent with a substantially unstructured peptide region, but for water-TFE $\chi_{TFE} \geq 0.1$, spectra for the urethane and those for Ac-Hel-Ala$_6$-OH are similar. These data suggest that substitution of a urethane for the acyl of Ac-Hel diminishes the capping efficiency relative to Ac but does not significantly change its character.

As evident in Figure 4c−e, more dramatic changes are seen for the remaining members of the X-Hel series. These spectra exhibit only six detectable lines, implying that at the temperature of the study, only one averaged conformational state is detectable. As noted above, this is the anticipated result for the urea and sulfonamide. The observation of a six-resonance NH spectrum for the *N*-pivaloyl-Hel peptide Figure 4c must reflect an anomalously rapid interconversion of the s-cis/s-trans pivalamide conformers. A likely explanation is the extreme crowding of the planar conformations of tertiary pivalamides relative to the nonplanar transition state for interconversion.

The spectrum of the sulfonamide, Figure 4d, with $\chi_{TFE} \geq 0.1$ shows a tight cluster of four rather than five NH resonances in the δ range of 7.68−7.78 ppm and further differs from the spectrum of Ac-Hel-Ala$_6$-OH in exhibiting a pair of resonances corresponding to two protons that shift by >0.4 ppm upon TFE titration.

The final example in this series is the urea, which yields the series of spectra shown in Figure 4e. At high χ_{TFE} five NH resonances are grouped in the helical δ range of 7.65−7.9 ppm, but one is found at a nearly TFE-invariant δ value of 8.3 ppm. In other contexts we have previously noted that a peptide NH H-bonded to a urea is significantly deshielded relative to the expected value for an amide.[19] Though other interpretations are possible, the data for the urea and sulfonamide suggest that unless conformations of the NH-1 and NH-2 H-bonds are optimal for helix extension, the strength of these bonds may be a counterproductive element for an X-Hel candidate.

The CD spectrum of Ac-Hel-A$_6$-OH at 25 °C in water-TFE $\chi_{TFE} = 0.2$ is consistent with the presence of weak helical structure,[20] showing an ellipticity minimum at 206 and 226 nm with approximately equal per residue molar ellipticities of -10×10^3 (deg·cm^2)/dmol and a steeply rising positive ellipticity at 195 nm of ca. 8×10^3 (deg·cm^2)/dmol. In pure water, the experimental ellipticity[21] is dominated by random coil and template CD contributions, and although negative below 250 nm, lacks distinctive helical features. Similar CD spectra are seen for the X-Hel-A$_6$-OH species with X = pivaloyl and mesyl, either in water or in water-TFE $\chi_{TFE} = 0.2$.

Even strongly capped, the five-residue helix of A$_6$-OH is expected to lie at the lower limit of CD detectability, and solubility prevents further extension of the length of an Ac-Hel-A$_6$-OH series. A more sensitive CD test of helicity was provided by extending the chain to eight amino acid residues with the help of a solubilizing lysine. CD spectra for the peptide conjugates Ac-Hel-A$_4$KA$_3$-NH$_2$, H-Hel-A$_4$KA$_3$-NH$_2$, and CH$_3$SO$_2$-Hel-A$_4$KA$_3$-NH$_2$, in water and in water-TFE, $0.05 \leq \chi_{TFE} \leq 0.15$, are shown in Figure 5a−c, and landmark features of the H, Ac, pivaloyl, sulfonamide, and urea derivatives X Hel-A$_4$KA$_3$-NH$_2$ are shown in Table 1 (data for the Ac-βAlanyl derivative **2g** and the Ac-Glycyl derivative **2h** are also included and will be discussed in a later section of this report).

Table 1. CD Residue Molar Ellipticities at 2 °C for TFE Titrations of X-HelA$_4$KA$_3$-NH$_2$ (**2a**−**g**) and X-A$_4$KA$_3$-NH$_2$ (**3**) in Water

		$-[\theta]_{222}$[b]		$-[\theta]_{208}$[b]		
X[a]	entry	water	15 mol % TFE	water	15 mol % TFE	isoellipsoidal point [nm]
H	**2a**	0		1		203
Ac−PP	**3**	−2		−2		203
Piv	**2b**	−4	−16	−3	−18	202
Moc	**2c**	−6	−22	−11	−20	203
Mes	**2d**	−7	−17	−8	−20	202
DMU	**2e**	−8	−19	−8	−20	202
Ac	**2f**	−12	−21	−11	−21	202
AcβAla	**2g**	−11	−18	−9	−18	202
AcGly	**2h**	−13	−22	−11	−20	202

[a] Abbreviations: Ac-PP = *N*-Acetyl-prolyl-prolyl; Piv = pivaloyl; Moc = methoxycarbonyl; Mes: Mesyl; DMU = dimethylcarbamoyl; Ac = acetyl. [b] Per residue elipticities in ((deg·cm^2)/dmol residue) $\times 10^{-3}$.

Figure 5a shows a TFE titration series for the reference conjugate Ac-Hel-A$_4$KA$_3$-NH$_2$. Its CD spectrum in water alone is clearly partially helical, and the addition of TFE acts to enhance the initial helicity. How are the features of this series changed if the acetyl cap is deleted? The TFE titration series of Figure 5b addresses this question[1,22] and a very similar CD series is observed for the TFE titration of Ac-Pro-Pro-A$_4$KA$_3$-NH$_2$, which contains an N-terminal H-bonding acceptor but lacks the conformational constraint that enforces a helical conformation on the Pro-Pro sequence. These two spectra closely resemble the temperature-dependent CD series observed for unordered peptides,[23] with the significant difference that the plateau or weak extremum observed for $215 \leq \lambda \leq 220$ nm is positive at low temperatures for the latter and negative at high χ_{TFE} for the former. A satisfactory N-cap and an internal constraint that imposes a helical conformation on the Pro-Pro sequence are necessary conditions for N-terminal peptide helix initiation by a member of the X-Hel family.

The TFE titration curve shown in Figure 5c for the CH$_3$SO$_2$-Hel-A$_4$KA$_3$-NH$_2$ conjugate differs from that of Figure 5a but closely resembles those observed for the urethane, urea, and pivaloyl congeners (not shown; see Supporting Information). Although the CD spectra taken at high χ_{TFE} are helical, the minimum at 222 nm is invariably less intense than that at 208 nm, and more significantly, although a negative ellipticity is observed as a shoulder at 222 nm, the features of the CD spectrum in water alone only suggest helicity. A detailed quantitative comparison of the six capped A$_4$KA$_3$-NH$_2$ sequences is found in Table 1. It is clear from this comparison that the helix-initiating capacity of Ac-Hel is matched by none of the nonamide H-bonding replacements that have been reported in this section. With the exception of the replacement of the acetamido function of Ac-Hel by an *N*-sulfamate,[24] results with which will be reported elsewhere, it appears that for Hel X-caps, despite its suboptimal features, a simple acetamide is superior to uncharged nonamide functionalities. This point is

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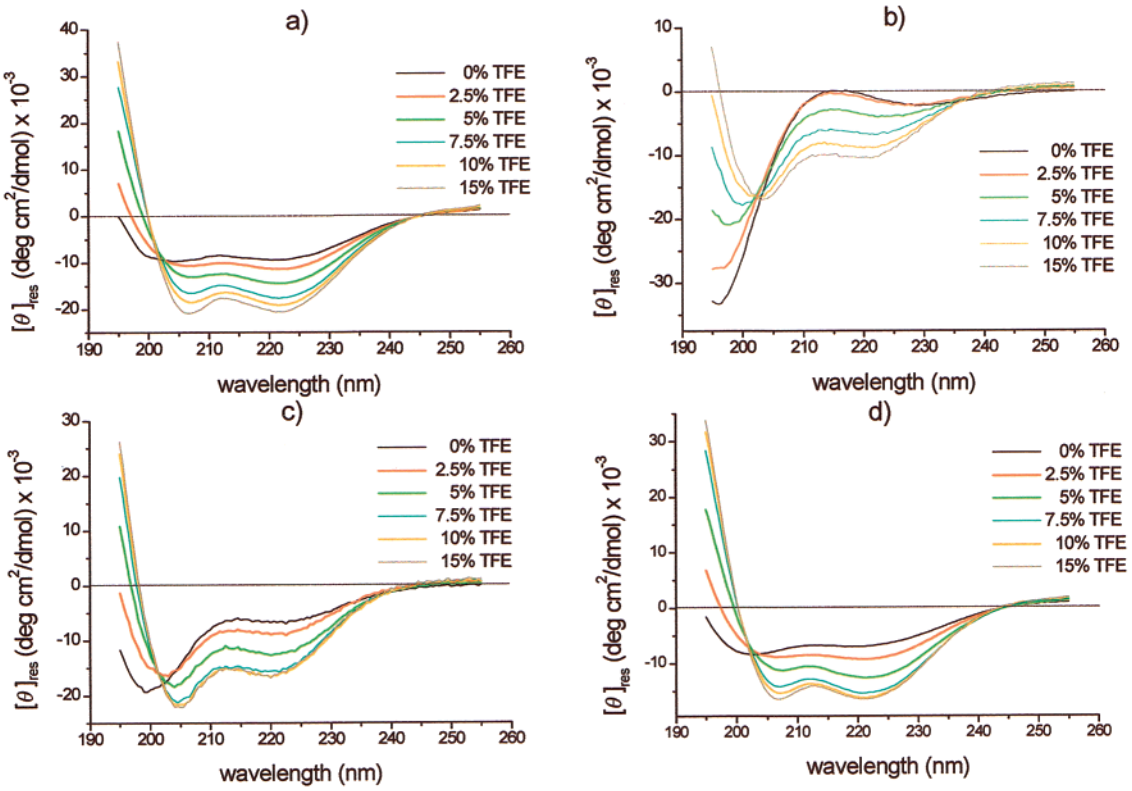

Figure 5. CD spectra for (a) Ac-HelA_4KA_3-NH_2 (**2f**), (b) H-HelA_4KA_3-NH_2 (**2a**), (c) Mes-HelA_4KA_3-NH_2 (**2d**), and (d) AcβAla-HelA_4KA_3-NH_2 (**2g**) in water and water/TFE−mixtures (mol % TFE given) at 2 °C.

underlined if one compares the titration curves of Figure 5a (X = Ac) and Figure 5d (X = AcβAla). The latter is one of the least efficient *N*-acyl caps introduced in Table 2 in a later section, but is clearly similar to acetyl in CD-derived helicity.

NMR Properties of Acyl-Derived X-Hel Caps

We have seen in Figure 4a that for Ac-Hel peptide conjugates an increase in helicity is mirrored by an increase in t/c, the ratio of integrated t and c state ^{1}H NMR resonances. Table 2 contains our examples for peptide conjugates of N-caps X-Hel, where X is a simple acyl function. For all such cases that we have examined, detectable t and c state resonances appear in ^{1}H NMR spectra. Can these resonances be used as helicity reporters?

Ease and accuracy of measurement provide the first criteria for answering this question. Many of the X-Hel caps of Table 2 are unsuited for t/c quantitation owing to peak overlaps between X-derived resonances and peptide CH resonances. For example, though spectra were consistent with their presence, t and c resonances could not be easily integrated for Ac-β-Ala-Hel-NH_2. An X-Hel candidate may also be unsuitable for t/c quantitative analysis of helicity if it has too narrow a range of t/c values, if its sensitivity to helicity changes is too high or too low, or if the limiting CD spectra for helix nucleating and nonnucleating states of X-Hel itself cannot be easily deconvoluted from models.

The useful range of t/c is an important issue because t/c is defined as a ratio of integrated NMR resonance intensities, and for optimum accuracy its useful range is normally $0.1 \leq t/c \leq 10$. Since t/c increases with length of a peptide and the helical propensities of its component amino acids, the t/c value for X-Hel-NH_2 sets an approximate lower bound on t/c for analogous X-Hel peptide conjugates. A preliminary analysis of these issues has been carried out with X-Hel-NH_2 **4**, X = formyl, chloroacetyl, methoxyacetyl, and *N*-acetylglycyl.

For X-Hel-NH_2, X = chloroacetyl and acetylglycine, t/c approaches 3, narrowing the useful t/c window. For a particular X-Hel-NH_2 and change of χ_{TFE} in the range 0 to 0.1, the change in mole fraction of the c state = 1/(1 + t/c) provides an approximation to the responsiveness of t/c to the intrinsic helicity of an X-Hel peptide. This change, which is 33% for X = Ac, falls in the range of 25−28% for X = methoxyacetyl, chloroacetyl, and formyl, and has an anomalously low value of 15% for X = Ac-Gly. Therefore formyl appears to be the viable alternative to acetyl.

A second important question concerns the correction of Hel-peptide CD spectra for cap effects. For X-Hel capped peptides containing 10 or more amino acid residues, where X is a simple acyl function, the contribution of the cap to the CD spectrum of the conjugate is usually insignificant. For shorter helical peptides, a cap correction is needed. Studies with the Ac-Hel conjugates[24] have shown that the CD spectrum of the (cs + ts) state of the template is characterized by a maximum at 195 nm $[\theta]_{max,molar} = +90\,000$ (deg·cm^2)/dmol, steeply declining through zero at 207 nm to a minimum at 213 nm $[\theta]_{min,molar} = -45\,000$ (deg·cm^2)/dmol. The helix-nucleating te state is significantly reduced in intensity at all wavelengths, exhibiting a maximum at 195 nm, a weak minimum at 210 nm $[\theta]_{min,molar} = -7000$ (deg·cm^2)/dmol and a second slightly more intense minimum at 232 nm $[\theta]_{min,molar} = -12\,000$ (deg·cm^2)/dmol, which may correspond to the n−π* transition of the tertiary amides. The local t−c conformation of the *N*-acetyl group thus has a substantial influence on the CD properties of Ac-Hel, and the most rigorous cap correction algorithm for the CD spectra of Ac-Hel peptide conjugates requires subtraction of separate

contributions of the te and (cs + ts) template states, weighted by their respective mole fractions. For Ac-Hel-NH_2 in TFE−water mixtures, the TFE dependence of these mole fractions is easily calculated[24] from the Ac-Hel-OH t/c value, which corresponds to the limiting (cs + ts) state, and the TFE dependence of t/c values for Ac-Hel-NH_2. Linear regression of TFE-dependent CD spectra and mole fractions yields estimates of limiting te and (ts + cs) state spectra. CD comparisons of Ac-Hel-OH and Ac-Hel-NH_2 with the analogous X-Hel derivatives taken from the acyl and nonacyl X-caps show that the spectra are qualitatively consistent within the acyl family, but not for the nonacyl X-group. This point is reinforced by a comparison of the CD spectrum of Ac-Hel-OH with Mes-Hel-OH. The latter exhibits a nearly featureless CD spectrum of low intensity. Although a considerable variation in CD intensity was seen for X-Hel-NH_2 with the *N*-acyl caps = formyl, chloroacetyl, methoxyacetyl, *N*-acetylglycyl, *N*-acetylsarcosyl, and *N*-acetyl-β-alaninyl, the CD spectra in water generally resembled the spectrum assigned to the (cs + ts) state of Ac-Hel-NH_2. These derivatives did, however, differ dramatically in their responsiveness to the presence of TFE. Within this series, the responsiveness of Ac-β-Ala-Hel-NH_2 was most similar to that of Ac-Hel-NH_2, a more modest change was seen for X = chloroacetyl or formyl, while the CD spectra for X = Ac-Gly and Ac-Sar were insensitive within experimental error to the presence of TFE. This insensitivity prevents rigorous cap correction in these cases. Assignment of limiting CD spectra for cap te and (cs + ts) states for Ac-Hel-NH_2 requires relatively large correlated TFE-dependent changes in both CD spectra and t/c values.

Only the *N*-formyl X-Hel cap potentially meets the practical requirements for a new NMR-based X-Hel helix reporter function. Its most attractive feature is the unique chemical shift value for the formyl proton, which lies in the range of δ 8.2−8.4 ppm, overlapped only by c-state backbone NH resonances that can be completely deuterated prior to measurement of t/c.

Replacement of the Acetyl-Derived Tertiary Amide by Other Acyl and Amidoacyl Groups: CD-Derived Relative Helicities of X-Hel-Peptide Conjugates

Table 2 compares values of $-[\theta]_{222}$ measured in water at 2 °C for sixteen acyl or amidoacyl functions X-Hel, linked to two different peptide sequences. The percent change in $-[\theta]_{222}$ that results from the Ac to X substitution is given in column 5 of Table 2. This provides a measure of the relative helix-capping efficiencies of the test cases. These range from −28% to + 66% of the standards, demonstrating that appropriate acyl-derived X-functions dramatically improve the efficiency of helix-stabilizing X-Hel caps.

Any of these 16 caps exhibit helix-inducing properties that are superior to the nonacyl-derived examples considered earlier in this report. This point is evident from the comparison of the TFE titration curve of Figure 5d with those of Figure 5c. In water the conjugates of the peptide A_4KA_3-NH_2 with the X-Hel urethane, sulfonamide, and urea caps show an average $-[\theta]_{222}$ of 5×10^3 (deg·cm^2)/dmol, an improvement over the zero value seen for the noncapped analogue H-Hel-A_4KA_3-NH_2 (see Table 1 and Figure 5b), but half the average value seen for the simple amide caps of Table 2. In strong contrast to the nonamide derived caps, even a relatively inefficient cap, typified by the Ac-β-alanine example of Figure 5d, show CD spectra in water that are identifiably helical. Moreover, on average, the value of $-[\theta]_{222}$ doubles for peptide conjugates of the amide-capped X-Hel if TFE in the range $\chi_{TFE} = 0.1$ is added.

Table 2. Comparison of Helix Inducing Capacities of AcHel and XHel Caps for Two Peptide Sequences: X-HelA_4KA_3-^{t}LInp_2K_4W-NH_2 (Series 1) and X-HelA_8-^{t}LInp_2K_4W-NH_2 (Series 2)

entry	X[a]	series[b]	$-[\theta]_{222}$[c]	%[d]
		reference compounds		
5a	Ac	1	11.5	−
5b	Ac	2	15.6	−
		weak caps		
6a	ClAc	1	9.7	−13
6b	Pr	1	8.3	−28
6c	AcβAla	1	8.9	−23
		moderate caps		
7a	HCO	1	10.8	−6
7b	AcGly	1	12.4	+8
7c	SSGly	2	13.9	−11
7d	Lev	2	14.1	−10
7e	MeOSucc	2	13.8	−12
7f	Glut	2	14.2	−9
		strong caps		
8a	Succ	1	17.7	+54
8b	Succ	2	21.0	+35
8c	Mal	1	19.1	+66
8d	Mal	2	23.4	+50
8e	Ox	1	19.1	+66
8f	Ox	2	18.0	+15
8g	Glut	1	14.5	+26
8h	AcβAsp	1	16.8	+45
8i	AcβAsp	2	21.0	+35
8j	CHDC	1	15.2	+32
8k	CHDC	1	14.0	+22

[a] Abbreviations: HCO = formyl; Piv = pivaloyl; Ac = acetyl; ClAc = chloroacetyl; Pr = propionyl; Ac-β-Ala = *N*-acetyl-β-alanyl; AcGly = *N*-acetyl-glycyl; SSGly = WK_4Inp_2^{t}L-Glycyl; Lev = levulinate; MeOSucc = succinate methylester; Glut = glutarate; Succ = succinate; Ox = oxalate; Mal = malonate, Ac-β-Asp = *N*-Ac-β-aspartate; CHDC = *trans*-1,2-cyclohexane dicarboxylate. [b] Series 1: X-HelA_4KA_3-^{t}LInp_2K_4W-NH_2. Series 2: X-HelA_8-^{t}LInp_2K_4W-NH_2. [c] Per residue ellipticities in water at 2 °C ((deg·cm^2)/dmol residue) $\times 10^{-3}$. [d] Deviation in $-[\theta]_{222}$ from standard compound **5a** for Series 1 and **5b** for Series 2.

The X-Hel caps of Table 2 are grouped according to their CD-characterized helix-forming capacity as weak (**6a**−**c**), average (**7a**−**f**), and strong (**8a**−**k**), relative to the CD helicity of the Ac-Hel peptides **5a** and **5b**. Two of the three weak helix formers of Table 2 (13 to 28% reduction in $-[\theta]_{222}$]) result from replacement of acetyl by propionyl ($CH_3 \rightarrow C_2H_5$) and introduction of an electron-withdrawing atom ($CH_3 \rightarrow Cl\text{-}CH_2$). With a helix-forming capacity within experimental error of the standard, formyl ($CH_3 \rightarrow H$) is the viable replacement candidate within the first two sections of the table. Among the average helix formers with helix-forming capacity ca. 10% below that of Ac, two, Lev and Me-Succ, share a common structural replacement feature ($CH_3 \rightarrow Y\text{-}CO\text{-}CH_2\text{-}CH_2$), and a third, Glut ($CH_3 \rightarrow {}^-OCO\text{-}CH_2\text{-}CH_2\text{-}CH_2$) also falls in this class. The data for the series Form ~ Ac > Prop suggests that only the smallest of the simple acyl functions are fully functional as internal N-caps for Hel, since other properties of these groups, such as carbonyl oxygen basicity or acceptor H-bonding capacity, are not expected to vary significantly. The observation that carbon chains terminating in polar functions and containing two or three methylenes are superior to Prop as acyl caps suggests compensation by the polar groups for the presence of longer hydrocarbon functions.

The three uncharged functions of Table 2 that allow extension of the peptide chain in the N-terminal direction beyond the Hel moiety contain glycyl or β-alanyl residues. Significantly, the sequence Ac-G-Hel-A_4KA_3-NH_2 (**2h**) is more helical than the

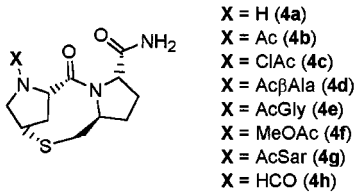

Figure 6. Modified Hel-NH_2 derivatives.

reference peptide **2f** (see Table 1). Owing to the presence of its lysine residue derivative **2h** is freely soluble and unaggregated in water, but experience with Ac-Hel conjugates suggests that substantial extension of the peptide sequence in either direction by nonpolar amino acid residues is likely to create solubility problems. Recently we have introduced spaced solubilizers as tools that permit the study of helical polyalanines, free of helix-modifying effects of charges.[25] The eighth Table 2 entry contains the sequence WK_4-Inp_2-^{t}L-G-Hel-A_8-^{t}L-Inp-NH_2 (**7c**), where Inp is 4-carboxypiperidine, an achiral γ-amino acid with rigid spacing properties, and ^{t}L is *tert*-leucine, with a side chain that selectively favors extended backbone conformations.[26] The presence of the Inp_2^{t}L sequence isolates the helix-inducing cap and its accompanying polyAla sequence from changes in helix stability induced by the positively charged polylysine N-terminus. The need for this spacer is demonstrated by the substantially reduced helicity ($-[\theta]_{222}$: 13900 → −9450; −32%) observed for the analogous sequence WK_4-^{t}L-G-Hel-A_8-^{t}L-Inp-NH_2 (data not shown, see Supporting Information) in which the rigid extender region Inp_2 is omitted. The ellipticity observed for the Gly-Hel cap for the spaced, solubilized derivative falls within 10% of the Ac-Hel standard, proving that for a strongly helix stabilizing cap of the X-Hel family one can extend the peptide sequence in the N-terminal direction.

The largest helical enhancements reported in Table 2 for Hel N-caps are seen for functions that bear negative charges. The simple series X = Ox, Mal, Succ, Glut examines the consequence of varying the carbon chain length that links the Hel-capping acyl function to a carboxylate anion. For peptide Series 1, which bears a positively charged lysine residue at site 5 of the octapeptide sequence, the helix stabilization order is Ox = Mal > Succ > Glut, with a maximum stabilization of +66% for Ox and Mal. To test whether a portion of this stabilization results from an electrostatic interaction between the negative and positive charges, we also studied a second series in which the C-terminal spaced solubilizing cap is applied to an octaAla sequence in which the peptide postive charge is absent (Series 2 of Table 2). Here the stabilization order is Mal > Ox ≥ Succ ≫ Glut, and a maximum stabilization of +50% is observed. This change suggests that a detectable electrostatic effect exists and is maximal for the *N*-oxalyl cap, yet for the malonate cap 75% of the stabilization observed with the A_4KA_3 sequence is retained with the A_8 sequence. An interesting detail is added by the last two entries of Table 2, which compare the helicities of peptides bearing an X-Hel cap, where a pair of diastereomers formed from *trans*-1,2-cyclohexanedicarboxylic acid. These are analogues of the succinyl cap; the smaller value of $-[\theta]_{222}$ relative to succinate seen for both isomers doubtless reflects the presence of the hydrophobic cyclohexyl moiety, but the significant difference between their $-[\theta]_{222}$ implies that in the chiral environment of the helix N-terminus, a relatively precise

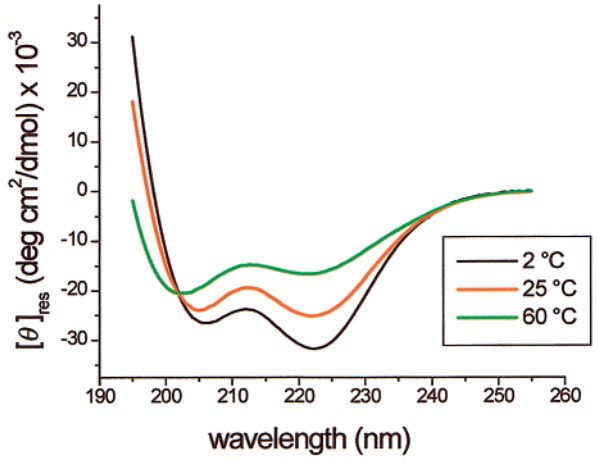

Figure 7. CD spectrum of WK_4Inp_2^{t}L-βAsp-Hel-A_{12}-^{t}LInp_2K_4-NH_2 at different temperatures in water.

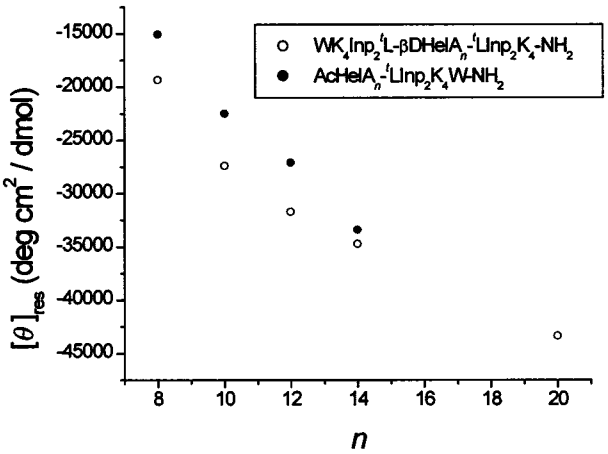

Figure 8. Comparison of $[\theta]_{222}$ for polyalanines N-capped by βAsp-Hel (open circles) and AcHel (closed circles). CD spectra were measured in water at 2 °C.

orientation of the charged carboxylate is required to achieve maximal helix stabilization.

It was important to establish that anion-derived cap stabilization can also be achieved with a Hel cap that permits N-terminal extension of the peptide chain. These aims are fulfilled by the β-amide derived from *N*-acetyl aspartic acid. Relative to the corresponding Ac-Hel analogues, the conjugate of Ac-β-aspartyl-Hel (β-Asp) with C-capped A_4KA_3 is 45% more helical, and its A_8 analogue is +35% more helical. Figure 7 shows CD spectra at three temperatures of the corresponding A_{12} peptide, WK_4-Inp_2-^{t}L-βAsp-Hel-A_{12}-^{t}LInp_2K_4-NH_2. For a helix of this length or larger, the contribution of capping residues to the overall ellipticities are small except at wavelengths below 210 nm. Although the low-temperature enhancement of the 222 nm minimum relative to that at 208 nm that has been noted previously[3] is evidenced in this series, these CD spectra are in other respects unremarkable and in accord with literature models.

Figure 8 plots the values of $-[\theta]_{222}$ for members of a homologous series of N- and C-solubilized, spaced β-D-Hel-A_n peptides, $8 \le n \le 20$, with the corresponding reference series for C-solubilized Ac-Hel-A_n peptides, $8 \le n \le 14$, where 14 is the maximum length of the solubilizing effect if only the C-terminal polylysine cap is present. The dramatic effect of the N-terminal negative charge is evident throughout the series, as is the convergence of the series at large *n*, which is predicted by Lifson−Roig modeling of these peptides with plausible

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initiation, propagation, and capping parameters. The differences in N-capping efficiencies of the two templates are maximal for short peptides, and become less significant for longer peptides in which spontaneous initiation of partially helical conformations within the central regions of the alanine sequences contributes significantly to the overall helicity.

Discussion and Conclusions

In this study, the N-caps X within the helical cap X-Hel of Figure 1 have been varied to maximize the efficiency of X-Hel as a helix-initiating peptide N-cap. The following points have been established. First, replacement of the acetamido function of the reference cap Ac-Hel by a urethane, a sulfonamide, and a urea has shown that these derivatives retain the capacity to initiate helices in test peptides to which they were N-terminally linked, and NMR evidence suggests that all form the required intramolecular H-bonds with the backbone NH donors of the peptide sequence. However, none of these three uncharged candidates for nonamide H-bonding acceptors is as efficient as a simple amide.

Among the simple amide candidates, the acetyl and formyl caps proved superior. Three effects could contribute to their primacy. The H-bonding efficiency of amides has been shown to correlate with the basicity of the amide carbonyl oxygen.[12] Inductive withdrawal of electrons from the amide function by α-substituents is expected to reduce this basicity. This is a plausible explanation for the superiority of Ac and Form over the peptide amide carbonyl. From comparisons of pK_a data for the parent carboxylic acids, the α-amido function of a simple N-capped amino acid residue is expected to be approximately as electron withdrawing as an α-chloro group. An inductive effect cannot explain the deficiencies of the propionyl group, nor the superiority of the *N*-acetylglycine group relative to other *N*-acetylated amino acids that lack polar side chains. Two other effects must be invoked to explain these cases.

A second-order hydrophobic effect may account for the propionyl result. The high hydration of an N-terminus of a helical peptide is expected to be sensitive to the intrusion of hydrophobic elements in proximity to a site of H-bonding. Such an effect might bias the rotamer population at the propionate CH_3-CH_2 bond, favoring conformations in which the methyl is oriented away from the N-terminus, and entropically disfavoring the flexible propionate over rigid functions with smaller hydrophobic character.

All other acyl caps in this study introduce polar hydrogen bonding functions within the vicinity of the carbonyl oxygen of the acyl function. Although the issue is complex and difficult to model, the most recent calculations for the preferred conformers of α-amino acid residues in water suggest the C_5 and PII conformations as important contributors.[27] The H-bond acceptor strength of an amide carbonyl oxygen is expected to be weakened if the oriented dipoles of neighboring H-bond donors are proximate[28] as with the C_5 conformation, or if they are capable of generating oriented waters within its proximity, which may occur with the C_7 and PII conformers. The unique behavior of glycine among the amino acids that lack polar side chains may reflect the much greater volume of its conformational space, which is expected to reduce the mole fractions of the ordered C_5 and PII conformers.

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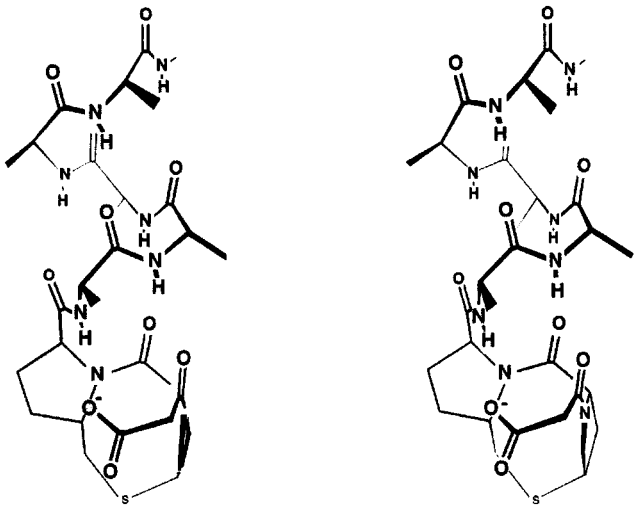

Figure 9. Stereodiagram of a MalHel-capped pentaalanine sequence, showing the H-bonding interaction between the malonat anion and the first NH of the peptide. The picture was drawn from a model that was generated with Quanta 98 from Molecular Simulations Inc.

In addition to embodying an acyl function linked to Hel, the negatively charged caps contain a terminal carboxylate anion. Does the molecular model proposed during the discussion of Figure 2b allow structural rationalization of the relative efficiencies of oxalate, malonate, succinate, and glutarate as H-bonding acceptor caps for Hel? A stereodiagram of an α-helix N-capped by malonyl-Hel is shown in Figure 9; malonate is optimal for the geometry proposed in this model.

As noted in the discussion of Figure 2b, a prediction of the model embodied in this figure is that replacement of the acetyl function of Ac-Hel by succinyl, forming succ-Hel, should induce a fractional increase in helical stabilization for a linked test peptide of similar magnitude to that seen for simple Ac or succinyl peptides. As noted in Table 2, the range of increase seen in the Hel series lies between 35 and 66%. Somewhat larger magnitudes were seen by Forood et al., and comparable magnitudes of stabilization for aspartate caps have been reported by Doig and Baldwin. At least as a working hypothesis, the best explanation for the increase in peptide helicity induced by *N*-succinyl and *N*-β-aspartyl caps is formation of an H-bond between a negatively charged oxygen of the carboxylate and the NH of the third residue of the peptide chain, allowing initiation of a helical conformation.

The most significant results of this study are the demonstrations that the same large stabilizations by negatively charged N-terminal caps operate for simple capped peptides and for X-Hel-capped peptides, and the observation that as with simple peptides substitution of a glycyl for an acetyl X-cap results in essentially no change in the helix-initiating propensity of X-Hel caps. Because the glycyl and the more efficient β-aspartyl-derived X-Hel caps allow extension of the peptide chain N-terminally through and beyond the Hel region, we have formally achieved the goal of constructing N-terminal helix stop signals that can be introduced anywhere within a peptide sequence. The exceptional efficiency of the β-aspartyl-Hel caps as helix initiators and the demonstration that the spaced solubilizers reported previously for polyalanines operate efficiently for X-Hel-peptide conjugates permits study of a variety of novel and versatile water-soluble, unaggregated polypeptides. We are currently investigating the promise of these species as CD helicity standards and will report on this work subsequently. Beyond these obvious direct applications, the working hypothesis that we have developed in this study concerning the

H-bonding requirements for N-terminal helical initiation, together with our recent observations of potent helix-enhancing effects of certain anions,[29] are likely to provide models for key aspects of the early folding events of globular proteins.

Experimental Section

Synthesis and Purification of Peptides. Peptides were prepared on a 0.025 mmol scale by automated continuous flow solid-phase synthesis on a PE Biosystems Pioneer Peptide Synthesizer with standard 9-fluorenylmethoxycarbonyl (Fmoc)/HATU chemistry. Fmoc-amino acids (>3 equiv) were weighed into labeled, letter-coded, 13 × 100 mm test tubes; HATU (<3 equiv, <28.5 mg) was then added to each test tube. For coupling of the helix template FmocHel-OH (see Supporting Information) only 1.5 equiv of the template and HATU were used and a catalytical amount of DMAP was added. The coupling time was prolonged to 60 min. For the attachment of capping groups to the template a double coupling step was applied, and a catalytical amount of DMAP was added. The coupling time was also prolonged to 60 min. Fmoc removal (deblock) was accomplished using a 1:1:48 (v:v:v) mixture of DBU:piperidine:DMF[30] for 6 min at a flow rate of 6 mL/min directly to waste and was monitored by integrating over time the UV absorbance of cleaved Fmoc chromophore.

Peptides were cleaved with 7 mL of a cocktail prepared directly before usage consisting of 82.5% TFA, 5% phenol, 5% water, 5% thioanisole, and 2.5% 1,2-dithioethane (v:v:v:v:v) for 2 h under an argon atmosphere and precipitated by dropwise addition to 35 mL of cold diethyl ether in a 50 mL polypropylene (PP) conical tube. The cold ether slurry was allowed to warm to room temperature over 30 min, at which time it was placed in a centrifuge at 3.5×10^3 revolutions per min (krpm) for 8 min. The precipitate was washed three times with ether, the liquid was decanted, and the conical tube was closed and vortexed for <1 min, until the solid clump loosened. After the final wash was decanted, the conical tube was closed halfway and the peptide placed under vacuum and dried at least 10 min. The crude, pale brown or white solid peptide was dissolved in 18.2 MΩ water and purified by HPLC.

Preparative HPLC separations were performed on a Rainin Dynamax SD-200 system equipped with a two-channel Rainin Dynamax UV-D II detector and Rainin Dynamax HPLC Method Manager software. Analytical HPLC separations were performed with a Waters 490 Programmable Multiwavelength Detector with a Waters 600E System Controller and a Waters 746 Data Module. All separations involved a mobile phase of 0.05% TFA in water (solvent A)/0.042% TFA in acetonitrile (solvent B). HPLC output was monitored at 214 and 280 nm.

Crude separations were performed with a Waters DeltaPak C_{18} 300 Å, 19 × 300 mm stainless steel column equipped with a short, hand-packed C_{18} (EM Science) guard column.

Analytical HPLC characterization was performed with a YMC ODS-AQ 200 Å, 4.5 × 150 mm stainless steel column equipped with a similarly packed 4.5 × 20 mm guard column.

Fine preparative separations were accomplished with a YMC ODS-AQ 200 Å, 10 × 150 mm stainless steel column equipped with a similarly packed 10 × 20 mm guard column. The criterion for purity was a single, symmetrical peak when rechecked by analytical HPLC. Purified peptides were lyophilized, transferred to 15 mL tared PP conical tubes, then lyophilized from 18.2 MΩ purified water and stored in PP conical tubes at 0 °C. Solutions of the peptides (see below) were also stored frozen in PP conical tubes.

Characterization. CD experiments were performed on an Aviv 62DS spectrometer equipped with a thermoelectric temperature controller and an Osram XBO 450-W high-pressure xenon lamp. CD data were collected after a 12 min temperature equilibration as an average of five scans at 1.0 nm bandwidth and 0.5 nm step size. After blank correction, spectra were smoothed with Aviv 62DS version 4.0s software polynomial fitting; high wavelength (250−255) intensities were close to zero and were therefore left uncorrected. UV spectroscopy was performed on a double-beam Varian Cary 100 Bio UV−visible spectrometer. Single-wavelength UV data were taken as an average of 5 scans. UV and CD cells (Hellma, QS, strain-free suprasil) were cleaned prior to each use first with water, followed by drying under vacuum, then with concentrated sulfuric acid that was drained without dilution, then again with purified water, followed again by drying under vacuum. The outsides of the windows were then wiped with HPLC grade methanol immediately prior to placement in the spectrometer. Blank UV and CD scans with the appropriate solvents were taken immediately prior to use of each cell. After a preliminary full UV scan (190−340 nm) indicated that only Trp absorbance was present, 5 scans at 280 nm were taken. Concentration was determined by the UV absorption of tryptophan[31] with $\epsilon_{280} = 5.56 \times 10^3\ M^{-1}\ cm^{-1}$ after taking a full UV scan of the CD sample (190−340 nm); 5 UV scans at 280 nm were taken. CD spectra were then obtained as indicated.

Samples were generally prepared for CD measurement by dilution of aqueous stock solutions to a concentration of 13 μM. Stock solutions were prepared by dissolving purified peptides (5−15 mg, 1−15 μmol) in a precisely measured (by mass; generally ∼10 mL) amount of 18.2 MΩ water or buffer in 15 mL PP conical tubes.

Electrospray mass spectroscopy (ESMS) of CD samples in pure water taken on a Waters Micromass ZMD 4000 yielded mass to charge (*m*/*z*) ratios within 0.75 mass units of the expected values and in each case showed several peaks corresponding to a range of values of *z* for a single value of *m*.

Materials and Abbreviations. All purchased chemicals were used as received. Chemicals were purchased from the following suppliers: amino acids and their derivatives from Novabiochem; biosynthesis grade *N*,*N*-dimethylformamide (DMF), high performance liquid chromatography (HPLC) grade methanol, and anhydrous diethyl ether from EM Science; trifluoroacetic acid (TFA) from Advanced ChemTech; HPLC grade acetonitrile from Mallinckrodt; 4-(Dimethylamino)pyridine (DMAP), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), piperidine, and diisopropylethylamine (DIEA) from Aldrich or Alfa Aesar, interchangeably; [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate] (HATU) and polystyrene (PS) functionalized with poly(ethylene glycol) (PEG) and PE Biosystems' Peptide Amide Linker (PAL) from PerSeptive Biosystems (PE Applied Biosystems). Water was purified and deionized to 18.2 MΩ by using a Millipore Gradient A10 water purification system equipped with a total organic carbon (TOC) monitor reading under 5 parts per billion (ppb). Deuterium oxide (D, 99.9%) was used as received from Cambridge Isotope Laboratories, Inc.

NMR. NMR spectra were obtained on a Varian Inova-500 MHz spectrometer. Chemical shifts are reported in ppm and are referenced to TMS (organic solvents) or TSP (water).

Acknowledgment. Generous financial support is acknowledged from NIH grant GM13453, NSF grant 9727356-CHE, and Pfizer Research. W. Maison acknowledges a Feodor Lynen Scholarship of the Alexander von Humboldt-Stiftung. P. Renold acknowledges a Scholarship of the Swiss National Science Foundation.

Supporting Information Available: Synthesis and analytical data of X-Hel derivatives and analytical data for peptides (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA010812A

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