The Structure and Energetics of Helix Formation by Short Templated Peptides in Aqueous Solution. 2. Characterization of the Helical Structure of Ac-Hel_1-Ala_6-OH

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Abstract: Analysis of NOE cross-peaks in ^{1}H NMR ROESY spectra of Ac-Hel_1-Ala_6-OH in water and in water−trifluoroethanol mixtures demonstrates that the template Ac-Hel_1 initiates an α-helix in a linked hexa-Ala peptide. Other NMR parameters and circular dichroism spectra are consistent with the presence of an α-helix, frayed at the C-terminus. Effects of NaCl, urea, temperature, and trifluoroethanol on the stability of a short alanine helix are reported.

In suitable solvents including water and water−trifluoroethanol (TFE) mixtures, the acyl function Ac-Hel_1 induces a structure in many polypeptides to which it is N-terminally linked.[1−3] Ac-Hel_1 has been shown to comprise the cs, ts, and te conformational states of Figure 1.[3] [Glossary of terms: c and t refer respectively to the s-cis and s-trans orientations at the acetamide CO−N bond; s and e refer to the staggered and eclipsed orientations at the C8−C9 bond. The cs state consists of the manifold of all conformations in which the template Ac-Hel_1 has the acetamide c-orientation and the thiolactam s orientation. The ratio t/c is the ratio of integrated peak intensities for t- and c-state ^{1}H NMR resonances, measured as reported previously;[3] TFE is 2,2,2-trifluoroethanol.]

On the NMR time scale, the te and ts states of an Ac-Hel_1 peptide conjugate are in rapid equilibrium and correspond to a single set of t state resonances with chemical shifts and coupling constants that are abundance weighted averages of limiting ts and te values. The t and cs states interconvert by a slow amide bond rotation and correspond to separate NMR resonances. Only the te state induces a structure within Ac-Hel_1-linked peptides; from indirect evidence the cs and ts states are assigned as peptide random coils.[3] Selective stabilization of the te state peptide results in an increase in both the fractional te character of the t state and in the value of t/c, estimated as the ratio of integrated areas of t and c state resonances in the ^{1}H NMR spectrum. Ac-Hel_1 thus behaves as a reporting conformational template.[1,3]

In this paper we demonstrate that the structure induced by Ac-Hel_1 in the te state of an N-terminally linked peptide is an α-helix. In the accompanying paper (*J. Am. Chem. Soc.* **1996**, *118,* 4249−4255) we derive peptide s values from the dependence of t/c on peptide length. The three papers of this series[3] provide the foundation for all applications of Ac-Hel_1-peptide conjugates. Other reports examine properties of conjugates of alanine-lysine peptides,[4] quantitation of the helix-stabilizing effects of trifluoroethanol,[5] and host−guest studies leading to template-derived s values for natural and unnatural amino acids.

For a range of aqueous solutions, the template ratio [ts]/[cs] $= K_1$ has been shown to be invariant, and, for a particular peptide conjugate, eqs 1 and 2 relate the mole fractions of template conformational states to t/c.[3] Thus for Ac-Hel_1-A_6-OH in D_2O at 25 °C, t/c = 1.78 corresponds to a t state composition of 44% noninitiating ts and 56% initiating te and to an overall state composition of 36% cs, 28% ts, and 36% te where $K_1 = 0.79$ in water or water−TFE mixtures at 25 °C.

$$\text{fraction cs} = \frac{1}{(1 + \text{t/c})}; \quad \text{fraction ts} = \frac{K_1}{(1 + \text{t/c})}; \quad \text{fraction te} = \frac{(\text{t/c} - K_1)}{(1 + \text{t/c})} \quad (1)$$

$$\text{mol fraction te in t-state} = \frac{\text{t/c} - K_1}{\text{t/c}}; \quad \text{mole fraction ts in t-state} = \frac{K_1}{\text{t/c}} \quad (2)$$

Scope of This Report

In the preceding paper in this series we viewed the peptide portion of an Ac-Hel_1 conjugate merely as an agent that can induce conformational changes within the template. We now examine in detail the nature of the complementary conformational changes induced by Ac-Hel_1 within the te state of the peptide. This paper analyzes all experimental evidence pertinent to a demonstration that the peptide te state consists of an α-helix that is nucleated outward from the peptide-template junction. Also reported are temperature, salt, urea, and trifluoroethanol-dependent helicity changes. These variables have been reported to strongly influence the helicity of medium-sized polypeptides in water. NMR resonances relevant to these analyses have been assigned unambiguously from conjugates Ac-Hel_1-A_6-OH that are selectively deuterated in the peptide region.[1]

Simple peptides in water exhibit unambiguous helical character only if they contain more than ten amino acid residues, and such helices are usually modeled as complex, rapidly

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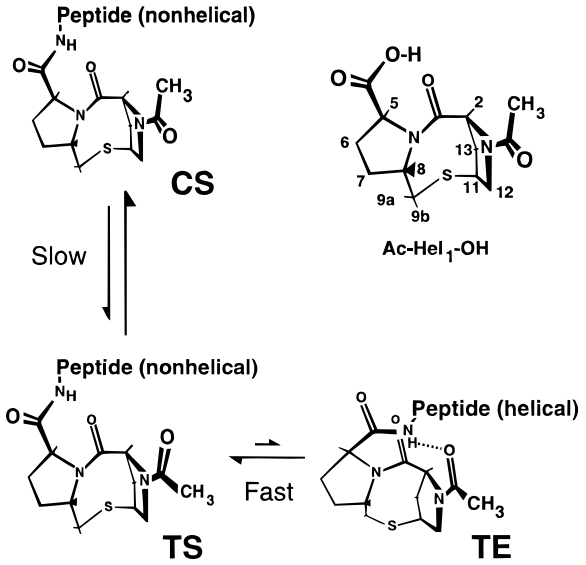

Figure 1. The three distinguishable conformational states of an N-terminal conjugate of the RCT Ac-Hel$_1$- with a polypeptide. Rates are referenced to the NMR time scale.

equilibrating mixtures of helical substates of varying length. Relative to these, a peptide helix formed by a conjugate such as Ac-Hel$_1$-A$_6$-OH is exceptional for its shortness and for its structural simplicity. Measurement of t/c permits calculation of the mole fractions of the nonhelical cs and ts peptide states, and as reported in the accompanying paper (*J. Am. Chem. Soc.* **1996**, *118*, 4249−4255), assignment of an average s value permits calculation of the relative abundances of the six substates within the te manifold. Monitoring the positional dependence of average helicity for this or similar conjugates should in principle allow a precise experimental characterization of the structure of this very simple helix. An s value near 1.0 and unique N-terminal helix initiation together imply that the fractional helicity at an amino acid site must decrease linearly with separation from the template-peptide junction. Outward from that junction the helix is progressively frayed.

The most incisive test of the positional dependence of helicity of a specific peptide cannot be used with Ac-Hel$_1$ derivatives. Relative rate constants for amide NH−ND exchange allow experimental characterization of the average solvent exposure of each amide proton of a peptide backbone.[6] At the pD of slowest amide exchange, the rate of equilibration between the t-state and the solvent-exposed c state for Ac-Hel$_1$-A$_6$-OH is twice the fastest NH exchange rate and ten times the slowest, and the exchange experiment therefore cannot provide an accurate measure of t-state amide exposure. Exchange measurements should however be applicable to peptides conjugated with analogous nonreporting templates that lack c-state equivalents.[7] We have previously studied fraying using a series of cognate peptide derivatives. Changes in t/c resulting from a glycine scan of Ac-Hel$_1$-A$_6$-OH are consistent with a monotonic, nearly linear decrease of the average site helicity with increasing separation from the template junction.[2]

The next section addresses two preliminary issues: aggregation and the structural uniquenesss of Ac-Hel$_1$. Many polypeptides form aggregates in water, resulting in substantial and selective stabilization of helical or other compact structures. A demonstration that Ac-Hel$_1$-peptide conjugates are completely monomeric within the concentration range of the study is an essential precondition for interpretation of helical stabilization data.

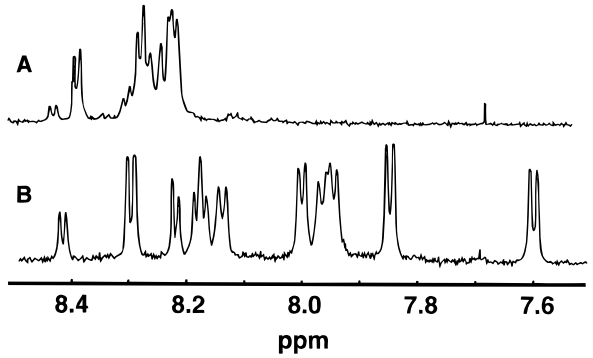

Figure 2. ^{1}H NMR spectra of N-capped Ala$_6$ peptides in H_2O-D_2O (9:1) in the NH resonance region. A. Spectrum of Ac-P-P-A$_6$-OH (300 MHz), the observed resonances fall in the random coil region (see text). B. Spectrum of Ac-Hel$_1$-A$_6$-OH (500 MHz) showing c-state NH resonances, δ 8.2−8.45, and t state NH resonances, δ 7.5−8.15.

The Ac-Hel$_1$ Analog Ac-P-P Does Not Induce Helical Structure in Peptides and Template-Peptide Conjugates Are Unaggregated in Water

The template Ac-Hel$_1$ is a conformationally constrained analog of the N-capped dipeptide acetyl-L-prolyl-L-proline. Does this simple dipeptide initiate structure-in linked peptides? Figure 2 compares the NH resonance regions of the ^{1}H NMR spectra of Ac-P-P-A$_6$-OH and Ac-Hel$_1$-Ac$_6$-OH in water. For the former, resonances appear in the range δ 8.2−8.5, consistent with random coil values of δ 8.1−8.8 for backbone amide NH functions and with the random coil value of δ 8.25 observed in water for alanine in the tetrapeptide GGAA.[8] By COSY and peak ratio analyses the c-state peptide NH resonances for Ac-Hel$_1$-A$_6$-OH are assigned to the range δ 8.15−8.45, within the random coil region. If the anomalous value[1] for NH-1 at the template junction is excluded from the t state resonances of δ 7.5−8.1, the resulting range δ 7.85−8.1 is consistent with values reported for small peptide helices.[9] A profound change in peptide conformational properties thus results from linking the proline residues of Ac-P-P-A$_6$-OH by the CH_2S conformational constraint of Ac-Hel$_1$.

Medium-sized peptides that lack charged side chain functionalities are often insoluble or undergo strong aggregation in aqueous solutions. To demonstrate the absence of significant aggregation, ^{1}H NMR spectra for dilution series of Ac-Hel$_1$-A$_6$-OH in D_2O were examined. Over a 500-fold concentration range with a lower limit of 23 μM, values of t/c were constant within the error limits of the analysis, and no significant changes in resonance position or width could be detected, as shown in Figure 3 which is typical for all spectroscopic regions. CD spectroscopy permitted a further dilution to 6.6 μM with insignificant change in CD ellipticity in the region of 195−250

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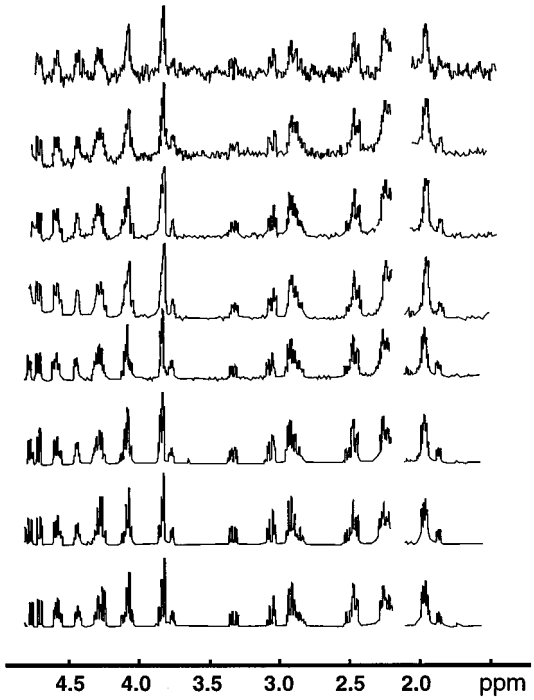

Figure 3. Concentration dependence of the ^{1}H NMR spectrum Ac-Hel$_1$-A$_6$-OH, 500 MHz, in D_2O in the region of conformation-sensitive template resonances. From bottom to top, the concentration range is 11.6 mM to 23 μM. Each successive spectrum is diluted by a factor of 2 but only eight of the ten spectra in the series are shown.

nm. NMR and CD spectra of dilution series for other Ac-Hel$_1$-peptide conjugates in water show similar invariance. As signaled by poor solubility and dramatic changes in ^{1}H NMR chemical shifts and line widths, the conjugates Ac-Hel$_1$-A$_2$-X-A$_3$-OH, X = I or F as well as higher alanine homologs of the unaggregated peptides Ac-Hel$_1$-A$_6$-OH, Ac-Hel$_1$-A$_6$-NH$_2$, Ac-Hel$_1$-A$_7$-K-NH$_2$ do aggregate at millimolar concentrations in water. We conclude, however, that no significant aggregation occurs in aqueous solutions of Ac-Hel$_1$-A$_6$-OH and its lower homologs at or below 20 mM. It is noteworthy that Ac-Hel$_1$ appears to confer water-solubility on its conjugates, perhaps reflecting the anomalous hydrophilicity of proline residues[10] and the polar effect of three proximate, aligned amide dipoles.

Circular Dichroism Spectra of Ac-Hel$_1$-Ala$_6$-OH

A characteristic ultraviolet circular dichroism spectrum is expected for a helical peptide in water, and fractional helical character is usually estimated from the magnitude of the experimental molar ellipticity at 222 nm.[11] This estimate is problematic for very short peptides, since theory predicts a significant length dependence for the helical CD spectrum,[12] and unambiguous experimental data are unavailable. Moreover, CD spectra of Ac-Hel$_1$-templated peptides must be corrected for the template substate ellipticities[3] as well as for ellipticity contributions of the random coil conformations of peptides in the cs and ts states. In this first report we explore the helical character of Ac-Hel$_1$-A$_6$-OH spectra that have been corrected only by dividing experimental molar ellipticities by the mole fraction of te state that is present and by the number of hydrogen bonds within the peptide helical region. More rigorous analyses

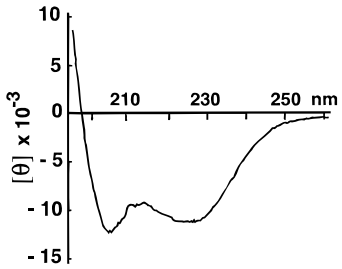

Figure 4. Circular dichroism spectrum of Ac-Hel$_1$-A$_6$-OH at 25 °C in 20 mol % TFE−H_2O, molar ellipticity per residue (deg-cm^2-dmol^{-1}), ordinate = molar ellipticity × (1/5) × (1/0.36). No correction has been made for the template or the random coil contributions (see text).

that quantitatively validate these assignments have been carried out and will be reported subsequently.

The CD spectrum of Ac-Hel$_1$-A$_6$-OH at 25 °C in H_2O is relatively featureless and uniformative, showing strong negative molar ellipticity that decreases from 195 to 250 nm, with a significant decrease in slope in the region of 222−228 nm and a per residue molar ellipticity at 222 nm of -2.2×10^4 deg-cm^2-dmol^{-1}. This is the expected CD spectrum under these conditions, since the 21% frayed fraction of the te state and the 64% of cs and ts states contribute substantial random coil character to the overall CD spectrum. The CD spectrum of an alanine random coil in these derivatives has been obtained by deconvolution;[13] it exhibits a negative per residue molar ellipticity that decreases steeply from 210 to 195 nm, as expected from literature values.[11] This ellipticity contribution together with the negative contribution of the template (ts + cs) composite state[3] in the region of 210−220 nm dominates the short wavelength CD spectrum of Ac-Hel$_1$-A$_6$-OH in pure water.

From the measured t/c value in 20 mol % TFE, the cs and ts states of Ac-Hel$_1$-A$_6$-OH must contribute only 6% random coil character to the CD spectrum, and the calculations of the accompanying paper (*J. Am. Chem. Soc.* **1996**, *118*, 4249−4255) show that the te state is substantially less frayed. The uncorrected CD spectrum for Ac-Hel$_1$-A$_6$-OH in 20 mol % aqueous TFE now shows the characteristic helical form throughout the spectrum, and the molar ellipticity at 222 nm lies within the range reported for shorter helical peptides.[14] Spectra for Ac-Hel$_1$-A$_6$-OH in a series of water−TFE mixtures exhibit negative ellipticities at 222 nm that correlate quantitatively with the mole fraction of the te state as calculated from t/c values (graph not shown).

Effect of Temperature and Concentrations of Salts, Urea, and Alcohols on the t/c Ratio for the Template-Peptide Conjugate, Ac-Hel$_1$-L-Ala$_6$-OH in Aqueous Solutions

The stabilities of helices formed in water from small peptides are usually strikingly dependent on temperature, and most show signififcant helicity only near 0 °C.[15,16] The negligible temperature dependence of t/c for the conjugate Ac-Hel$_1$-A$_6$-OH in water in the range of 10−60 °C is shown in Figure 5; similar temperature invariance was previously noted for the simple template amide derivative Ac-Hel$_1$-NHCH$_3$.[3] The failure to observe melting of the alanine te state is consistent with the resistance to melting noted by Doty and Gratzer for alanine-

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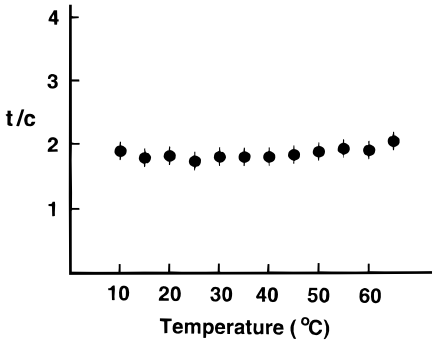

Figure 5. Temperature dependence of the t/c ratio for Ac-Hel$_1$-A$_6$-OH in H_2O-D_2O (9:1), pH 1, derived from 500 MHz ^{1}H NMR spectra. Error bars are shown where they exceed the dot boundary.

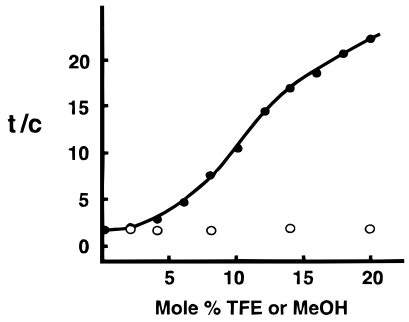

Figure 6. Dependence of the t/c value for Ac-Hel$_1$-A$_6$-OH on the mol fraction of TFE (CF_3CD_2OH, filled circles) and CD_3OH (open circles) ^{1}H 500 MHz NMR spectrum, H_2O-D_2O (9:1), pH 1, 25 °C.

block copolymers[16] as well as with the small temperature dependence reported by Scheraga et al. for the alanine s value obtained in an oligopeptide matrix.[18] Ooi and Oobatake have recently predicted the temperature dependence of the unfolding of short alanine helices, based on a protein-derived hydrophobic model.[19] Our data are inconsistent with their prediction, perhaps because polyalanine is the limiting case for their model. Large calorimetric $\Delta H°$ values have been reported by Baldwin et al. for the helix-coil transition of alanine-rich peptides.[20] The origin of these different temperature effects will be the subject of a subsequent report.

The urea-induced denaturation of proteins is usually attributed to a combination of interactions with both backbone and side chain functionalities of the amino acid sequence.[21] Helix bundles as well as isolated helices formed from medium sized peptides show marked reductions in fractional helicity in the presence of urea.[22] The effect of urea on t/c for Ac-Hel$_1$-A$_6$-OH corresponds to a fractional reduction of χ_{te} of 0.07. Relative to amino acids with bulkier side chains, the partitioning of alanine from water to 8 M aqueous urea is unusually small,[23] and, as with the temperature dependence, this modest effect of urea on the template state ratio is analogous to the insensitivity to urea-induced denaturation observed by Doty and Gratzer for an α-helical alanine block polymer.[17] The small effect of urea observed for Ac-Hel$_1$-A$_6$-OH is therefore consistent with the absence of urea-sensitive amino acids within the peptide sequence. It distinguishes the behavior of this peptide from the normal denaturation seen for peptides and proteins with heterogeneous amino acid sequences.

At high concentrations sodium chloride and other salts have been observed to stabilize small helices in water,[16,24] and for Ac-Hel$_1$-OH itself with two proximate and aligned amide dipoles, t/c doubles with 3 M salt is added to water.[3] For Ac-Hel$_1$-A$_6$-OH, a change from 0 to 3 M NaCl causes t/c to increase from 1.78 to 3.15, with the significant increase occurring above 1 M. The te state stabilizing effect of the alcohol trifluoroethanol (TFE) is much more dramatic, as seen in Figure 6. For a change from 0 to 10 mol % TFE at 25 °C for Ac-Hel$_1$-A$_6$-OH, the te mol fraction within the template t state increases from 56 to 93%. Methanol by contrast has no detectable effect as analogous concentrations. All water soluble alcohols including methanol enhance helicity, but their efficiency increases with alkyl chain length, and it is anomalously high for TFE.[5,14,25] Elsewhere we have analyzed the origins of this effect.[5]

Trifluoroacetic acid (TFA), which is added to samples to suppress ionization of Ac-Hel$_1$-A$_6$-OH, also selectively stabilizes the te state, causing a ca. 30% increase in t/c for a change from 0 to 2 mol %. Since TFA at this concentration range is largely dissociated, this te stabilization should probably be attributed to a simple salt effect, although a medium effect akin to that seen with TFE may also be operative. At much higher mole fractions of TFA, denaturing, helix-breaking effects are expected to dominate.[17]

^{1}H NMR Properties of the Peptide Backbone of Ac-Hel$_1$-Ala$_6$-OH in Aqueous Solution—$^3J_{NH\alpha}$ Coupling Constants

Signature ^{1}H NMR properties provide tests of the presence of helical conformations. Although the characteristic NOE interactions described in the next section provide the most incisive structural information, correlative structural data include solvent shielding of backbone NH functions and restricted backbone ϕ angles, reflected in reduced temperature dependences of NH chemical shifts and in small vicinal coupling constants $^3J_{NH\alpha}$.

NMR analysis in water and water−TFE mixtures of three selectively alanine-deuterated forms of Ac-Hel$_1$-A$_6$-OH has permitted assignment of NH resonances in c and t manifolds to specific alanine residues. Since the observed t state resonances for Ac-Hel$_1$-A$_6$-OH correspond to an s-e state average in water at 25 °C, coupling constants as well as chemical shifts and their temperature dependences are averages of limiting random ts and structured te values, weighted 41:59 or 1:1.44 toward the te state. The peptide within the structured te state also exhibits s value dependent fraying, implying that the fractional helicity at each amino acid residue is expected to decrease monotonically with separation from the peptide-template junction. Ac-Hel$_1$-conjugates exhibiting modest t/c (e.g., <4) will thus possess substantial random coil character.

For peptide derivatives, Karplus correlations of the vicinal coupling constant $^3J_{NH\alpha}$ with the peptide backbone dihedral angle ϕ have an extensive literature.[26] Although the ϕ value

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Table 1. Ac-Hel$_1$-A$_6$-OH NH Coupling Constants: $^3J_{NH\alpha}$ 9:1 H_2O-D_2O 25 °C

TFE (mol %)	$^3J_{NH\alpha}$ (Hz)					
	NH-1	NH-2	NH-3	NH-4	NH-5	NH-6
A. t State Resonances (±0.2)						
0	6.4	5.5	5.4	5.8	6.3	6.7
2.7	6.5	5.5	5.4			6.5
5.9	7.0		5.2			6.6
9.7	7.2	5.1	4.9	5.6	6.6	6.5
14.3	7.3	4.9	4.7	5.6	6.7	6.4
20.0	7.3	4.9	4.5	5.7	6.7	6.4
B. c State Resonances (±0.3)						
0	5.3		5.7			
2.7	5.5	5.4	5.8			6.5
5.9	5.4	5.4	5.9			6.6

of −57° calculated for the idealized Pauling α-helix corresponds to a $^3J_{NH\alpha}$ of 3.9 Hz, substantially larger values are usually observed for residues within helices of proteins with well-defined solution structures. Three consecutive peptide residues with a $^3J_{NH\alpha}$ <6.0 Hz has been offered as a criterion for local helicity.[26]

The data of Table 1 present $^3J_{NH\alpha}$ values for both c and t state NH resonances of Ac-Hel$_1$-A$_6$-OH in water and TFE−water mixtures at 25 °C; c-state resonances are not detectable at high χ_{TFE}, and peak overlap complicates assignments in water. A $^3J_{NH\alpha}$ value in water of 6.5 has been reported for an alanine residue flanked by G and A within an unblocked tetrapeptide.[8] In the alanine-rich environment of the c state of Ac-Hel$_1$-A$_6$-OH, a significantly smaller value is observed. We find no other c state NMR signature suggestive of nonrandom structure.

The t state resonances are well-separated and allow a more detailed analysis of variations of ϕ angle at the six NH sites of the hexaalanine function. In a t state helical structure, the backbone ϕ and ψ conformations of residues 1−5 are constrained by a flanking pair of amide−amide hydrogen bonds. Residue 6, by contrast, lacks backbone constraint and is expected to sample random coil ϕ and ψ angles. The observed $^3J_{NH\alpha}$ values of 6.4−6.7 δ are consistent with this expectation.

The environment at NH-1 can assume several 3_{10}-α hybrid geometries containing bifurcated hydrogen bonds in which the average hydrogen bond length varies significantly,[1,3] and measurements in organic solvents show a substantial variation of the $^3J_{NH\alpha}$ value at this site with changes in solvent polarity and peptide length.[1] Variations of χ_{TFE} clearly result in similarly large changes.

As expected for residues within a helix core, the $^3J_{NH\alpha}$ values for t state resonances at sites 2 and 3 show a progressive shift to lower values with increasing χ_{TFE}. For a helical structure, the ϕ angle at residue 5 is largely defined by the nature of the geometry of the last intramolecular hydrogen bond within the helix. The value observed for the t state $^3J_{NH\alpha}$ at site 5 suggests a special capping orientation for a residue at the helix C-terminus, and a similar value has been noted for the templated helices formed in organic solvents.[1] All $^3J_{NH\alpha}$ data are consistent with previous results observed for templated helices in organic solvents and with the helical structure assigned from NOE evidence as described below.

Table 2. Amide NH Temperature Dependences of Chemical Shifts for Ac-Hel$_1$-A$_6$-OH 9:1 H_2O-D_2O 5−60 °C

NH-1	NH-2	NH-3	NH-4	NH-5	NH-6
A. t State Resonances (−ppm × 10^{-3})					
3.4	5.2	5.7	4.3	3.9	4.9
B. c State Resonances (−ppm × 10^{-3})					
7.9	8.1	8.4	7.8[a]	7.8[a]	8.3

[a] Averaged owing to peak overlap.

Effects of Temperature and TFE Concentration on NH Chemical Shifts

An unusually small dependence of NH chemical shift on temperature has been widely used as a measure of the degree of shielding of an amide NH from solvation.[27] For chemical shifts in water of intramolecularly hydrogen bonded amide NH functions of highly constrained alumichromes,[28] average temperature dependences are -3.0×10^{-3} ppm/K, which increase to -4.0×10^{-3} ppm/K for less constrained β-turns.[29]

Data of Table 2 show temperature dependences of amide NH chemical shifts of Ac-Hel$_1$-A$_6$-OH in water over the range of 10−65 °C. The data establish a large difference between the degree of solvent shielding of the c and t state populations, and despite the presence within the t-state manifold of a significant fraction of the unstructured ts state, the observed t state temperature dependences are close to the above averages.

The change in the observed temperature dependence attributable to the presence of ts state character in the t manifold can be modeled by using the known ts−te mol fractions with the assumption that the temperature dependences of the ts state NH resonances are equal to those observed for the corresponding cs resonances. A te state temperature dependence at NH site n is then, $(TD_t - 0.41TD_c)/0.59$, where TD_t and TD_c are observed t and c state temperature dependences. The resulting te state values for $-(\Delta\delta/\Delta T) \times 10^9$ calculated for NH 1−6 are, respectively, 0.3, 3.2, 3.8, 1.9, 1.1, and 2.5. All lie in the range expected for solvent-shielded protons.

In the urea concentration range above 2 M, significant chemical shift changes occur for Ac-Hel$_1$-A$_6$-OH in water. All NH resonances are deshielded with added urea, although only modest changes are observed in c state resonances. The largest change in a t state resonance is seen for NH-1 (0.1 ppm), with smaller changes seen for the other NH resonances (0.06−0.08 ppm). These changes are consistent with the decrease in te state stability reflected in the t/c values.

Figure 7 depicts the large changes in chemical shift of the NH resonances of Ac-Hel$_1$-A$_6$-OH observed with progressive addition of the helix-stabilizing alcohol trifluoroethanol. The TFE-induced change in t/c shown in Figure 8 is also evidenced at the left of the spectrum of Figure 10 by the disappearance of easily detectable c state NH resonances at high TFE concentrations. With the exception of the t state resonance at peptide site 3, which shows a very small chemical shift change, all NH resonances move significantly upfield with increasing concentrations of TFE. The largest effect is the 0.9 ppm shift of the t state of NH-1. Insignificant solvent-induced chemical shift changes accompany the change in TFE concentration from 10 to 20 mol %, and this convergence is consistent with the high values for t/c. The t state composition in water alone is 44% ts and 56% te, which shifts to 3% ts and 97% te in 10 mol %

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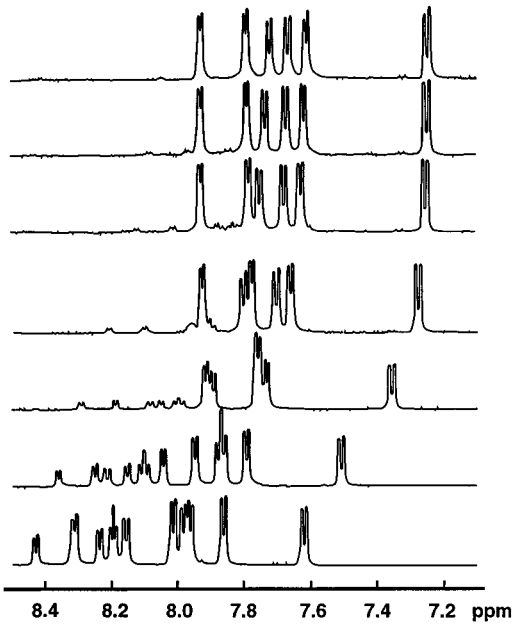

Figure 7. Effect of trifluoroethanol on the NH resonances of Ac-Hel$_1$-A$_6$-OH, 500 MHz, H_2O-D_2O (9:1), pH 1, 25 °C. From bottom to top, the mol fractions of TFE are, respectively, 0, 2.7, 5.9, 9.7, 14.3, 17.0, and 20.0 mol %.

TFE−water. Neglecting fraying and under the likely assumption that the effect of TFE on the solvent-shielded chemical shifts for the te state is negligible, one can approximate the chemical shifts for ts states by assuming that the observed t state chemical shift in water is a simple linear function of limiting te and ts chemical shifts, weighted by their t/c-derived mol fractions: $\delta_{obs} = 0.59\delta_{te} + 0.41\delta_{te}$ and that the te state chemical shift in water alone at each NH site is equal to the value seen at high TFE concentration. Then $\delta_{ts} = (\delta_{obs} - 0.59\delta_{te})/0.41$, and calculated values at sites 1, 2, 4, 5, and 6 are, respectively, δ 8.1, 8.3, 8.4, 8.4, and 8.8. These fall consistently in the random coil range of δ 8.1−8.8 that is also seen for the NH resonances of the cs state.

Studies of the temperature dependences of NMR resonances for amide NH functions has permitted an experimental distinction to be drawn between the degree of solvent exposure of cs and t state resonances, but helical fraying within the t state is not reflected detectably in the temperature dependencies, likely owing to the dominance of other local effects. The best experimental evidence for t-state fraying thus remains the large site dependent changes in t./c that are observed when a single helix-breaking glycine is substituted in turn for each of the alanine residues of Ac-Hel$_1$-A$_6$-OH.[2]

Structural Analysis of the Peptide Conformation of Ac-Hel$_1$-A$_6$-OH from Nuclear Overhauser Effects−Demonstration of the Helical Character of the te State

Among the three classes of polypeptide secondary structure, helices are the most compact and are characterized by an unusually large number of short and medium-range nonbonded distances between pairs of hydrogens. These generate a correspondingly large number of independent, structurally diagnostic nuclear Overhauser effects (NOEs) in the ^{1}H NMR spectrum of a helical peptide.[29] Viewed as constraints on the conformation of a flexible, linear peptide, these NOE-deduced interproton contacts differ dramatically in their structural significance, with the most distant NOE interactions as measured through covalent linkages providing the most compelling conformational evidence. The medium range $\alpha\beta$(i,i+3), αN(i,i+2), αN(i,i+3), and αN(i,i+4) NOE interactions which occur between proximate protons on residues of adjacent helical loops are exceptionally useful; these correspond to covalent separations of 12, 10, 13, and 16 bonds, respectively. Pertinent distances obtained by molecular mechanics modeling of both 3_{10} and α-helical conformations of Ac-Hel$_1$-A$_6$-OH are shown in Table 3.

An α-helix is distinguished from a 3_{10} by short $\alpha\beta$(i,i+3) contacts, and detection of strong $\alpha\beta$(i,i+3) interactions provides cogent evidence for α character. Although they can be difficult to detect at low sample concentrations, the relative intensities of αN(i,i+2), αN(i,i+3), and αN(i,i+4) medium range NOEs further define helical structure.[29] The αN(i,i+2) interaction is strong only for a helix with 3_{10} character, and the αN(i,i+4) interaction is detectable only for a helix with α character. An experimental distinction between 3_{10} and α-helical structure is important in the light of studies by Millhauser and co-workers using a novel spin labeling technique applied to short peptide helices. These studies have revealed 3_{10} character and the operation of a subtle balance between helical stability and the dominance of 3_{10} versus α structures.[31]

Other factors being equal, an NOE intensity is maximal for pairs of protons in van der Waals contact. An r^{-6} decrease in intensity is seen for an increase in the interproton distance r, but intensity is also dependent on local structural factors such as changes in internuclear vectorial motions.[30,32] The presence of a conformation of interest at a significant concentration in solution is demonstrated compellingly if all detectable NOE interactions correspond to proton contacts that lie within an experimentally detectable distance and if most of the calculated short conformational contacts correspond to observable NOE interactions. Under the conditions of our studies of Ac-Hel$_1$ peptide conjugates in water, we were unable to detect NOE interactions corresponding to interproton separations greater than 4.2 Å.

Three problems attended the NOE analysis of Ac-Hel$_1$-A$_6$-OH. First, the hexaalanine function exhibits peak overlap for most alanine α- and β-hydrogen resonances. Second, with a molecular weight of 724 D the template-peptide conjugate lies within the insensitive region for NOESY spectroscopy. Third, in pure water only 36% of Ac-Hel$_1$-A$_6$-OH is present as a te state, and fraying within the te conformational manifold is expected to further reduce the population of helical residues at sites distal to the template junction. The peak overlap problem was solved through synthesis and study of three selectively deuterated conjugates: Ac-Hel$_1$-404444-OH, Ac-Hel$_1$-440444-OH, and Ac-Hel$_1$-344144-OH, where "4" corresponds to NH-CD(CD$_3$)-CO, "3" to NH-CH(CD$_3$)-CO, "1" to NH-CD(CH$_3$)-CO, and "0" to undeuterated alanine. After attempts to utilize NOESY spectroscopy proved fruitless, ROESY spectra[33] were employed all for NOE analyses. Spectra were run in D_2O, in 9:1 H_2O/D_2O, and in water mixtures containing 9 mol % and 20 mol % TFE. As noted above, TFE shifts the template state population emphatically to favor the structured te state and

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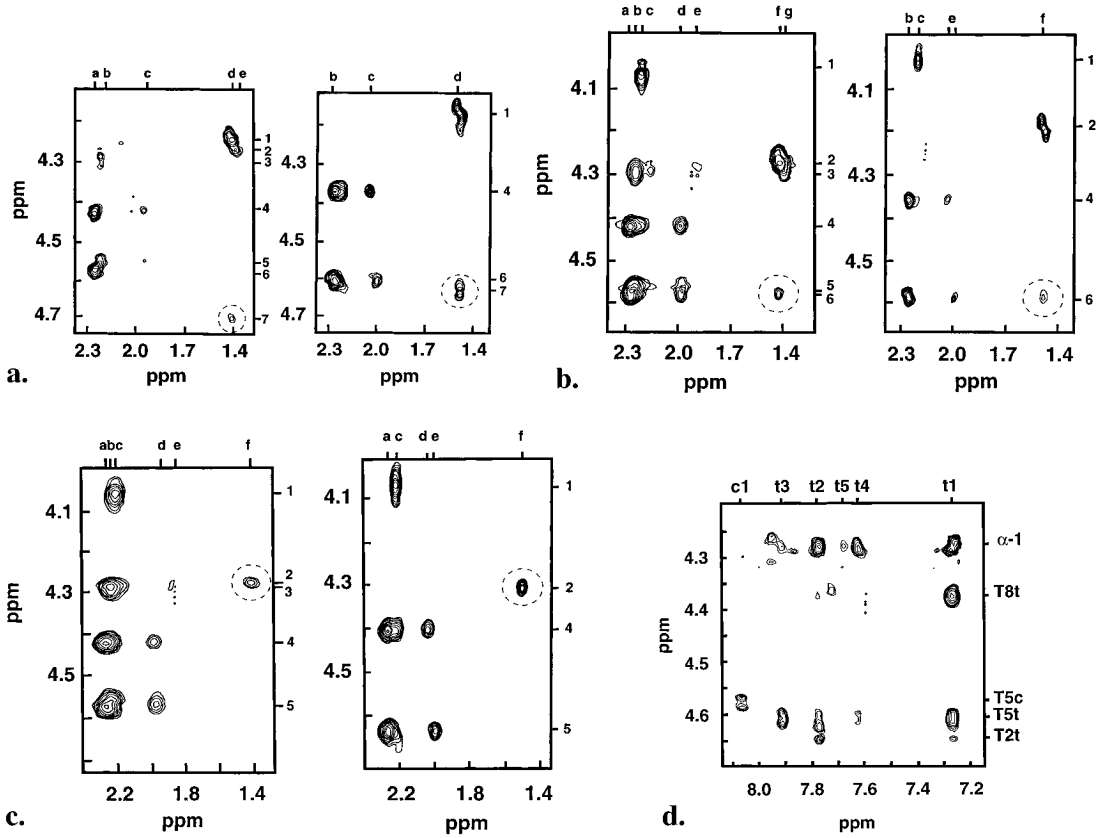

Figure 8. Slices of ROESY spectra, 500 MHz, pD 1, 25 °C, of Ac-Hel$_1$-A$_6$-OH derivatives, selectively labeled with deuterium in the peptide region. The $\alpha\beta$(i,i+3) crosspeaks are circled in the spectrum obtained in D_2O, left, and in 20 mol % TFE-d_3 −80 mol % D_2O, right. a. ^{1}H phase-sensitive ROESY spectrum of Ac-Hel$_1$-404444-OH (see text). The $\alpha\beta$(i,i+3) crosspeaks between the template C-2 and Ala-2 β-methyl protons are circled. Code for resonance assignment (-c and -t refer to c-t state assignment): a. H-6,7-c; b. H-6,7-t; c. Ac-CH_3-ct; d. Ala-2 CH_3-t; e. Ala-2 CH_3-c; 1. Ala-2 αCH-t; 2. Ala-2 αCH-c; 3. H-8-c; 4. H-8-t; 5. H-5-c; 6. H-5-t; 7. H-2-t. b. ^{1}H phase-sensitive ROESY spectrum of Ac-Hel$_1$-440444-OH (see text). The $\alpha\beta$(i,i+3) crosspeaks between the template C-5 and the Ala-3 β-methyl protons are circled. Code for resonance assignment: a & d. H-6,7-c; b & e. H-6,7-t; c. Ac-t; f. Ala-4 CH_3-t; g. Ala-4 CH_3-c; 1. H-12-t; 2. Ala-1 αCH-t; 3. H-8c; 4. H-8-t; 5−6. H-5-c-t. c. ^{1}H phase-sensitive ROESY spectrum of Ac-Hel$_1$-344144-OH (see text). The $\alpha\beta$(i,i+3) crosspeaks between the Ala-1 α-CH and the Ala-4 β-methyl protons are circled. Code for resonance assignment: a & d. H-6,7-c; b & e. H-6,7-t; c. Ac-t; f. Ala-3 CH_3-t; g. Ala-3 CH_3-c; 1. H-12-t; 2. Ala-3 αCH-t; 3. Ala-3 αCH-c & H-8c; 4. H-8-t; 5. H-5-t. d. ROESY spectrum of Ac-Hel$_1$-344144-OH in 20 mol % TFE-d_3 −80 mol % (9:1 H_2O-D_2O). Entries on the upper horizontal axis correspond to chemical shifts of one c-state and five of the six t-state NH resonances. Entries on the right vertical axis correspond to chemical shifts for the t-state resonances for the αH (α1) of Ala-1 and the template 2-, 5-, and 8-resonances. The template c-state 5-resonance also shows a crosspeak. From left to right, top to bottom, the significant resonances are t3-α1, αN(i,i+2); t2-α1, αN(i,i+1); t5-α1, αN(i,i+4); t4-α1, αN(i,i+3); t1-α1, αN(i,i); t1-T8t; c1-T5c αN(i,i+1); t3-T5t, αN(i,i+3); t2-T5t, αN(i,i+2); tr-T5t, αN(i,i+4); t2-T2t, αN(i,i+3); t1-T2t, αN(i,i+2).

Table 3. Interatomic Distances Calculated for Ac-Hel$_1$-A$_6$-OH by Molecular Mechanics

NOE interaction	covalent separation (no. of bonds)	α-helix (Å)	3_{10} helix (Å)
αN(i,i+1)	4	3.6	3.5
NN(i,i+1)	5	3.0	2.9
βN(i,i+1)	5	2.5	3.0
$\alpha\beta$(i,i+3)	12	3.0	4.7
αN(i,i+2)	7	4.7	3.6
αN(i,i+3)	10	3.7	4.0
αN(i,i+4)	13	4.1	6.7
NH-1, C-8	6	3.3	

increases the conformational homogeneity of the helical peptide in that state. It also enhances signal intensity by substantially increasing the solution viscosity.

NOE interactions within both c and t manifolds appear in the ^{1}H phase-sensitive ROESY spectrum of Ac-Hel$_1$-A$_6$-OH in 9:1 H_2O-D_2O. The c states show a very strong αN(i,i+1) crosspeak between the C-5 template α-H and the NH of the first alanine residue, but no crosspeak can be detected between NH-1 and the proton resonance corresponding to template H-8, suggesting that the helical ψ dihedral angle[3] at the C-5-CO bond is relatively unpopulated. Other than the local αN(i,i), $\alpha\beta$(i,i), and αN(i,i+1) interactions, which lack structural significance, no c state template-peptide or interpeptide interactions were detected, either in water or in TFE−water mixtures. The NOE spectrum of the c state peptide thus provides no evidence for significantly populated medium or long range structure.

The t state shows many structurally significant NOE interactions. The strong αN(i,i+1) crosspeak between the C-5 template α-H and the NH of the first alanine residue is accompanied by an equally intense crosspeak between NH-1 and the proton resonance corresponding to template H-8, consistent with a significantly populated helical ψ dihedral angle at the template C-5-CO bond. The expected ladder of short range NN(i,i+1) interactions characteristic of a helix are seen in water and in the two TFE−water mixtures. In all solvents the t state also shows three consecutive medium-range template-peptide and peptide−peptide $\alpha\beta$(i,i+3) NOE interactions characteristic of an α helix. (The deuterium labeling pattern required to detect the fourth Ala-2,Ala-5 $\alpha\beta$(i,i+3) interaction was not explored.) The three are shown in Figures 8 (parts a−c), in which portions of ROESY spectra in D_2O and in D_2O−20 mol % d_3−TFE are compared. As expected, the relative intensities of the $\alpha\beta$(i,i+3)

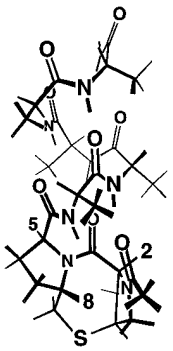

Figure 9. Molecular mechanics minimized structure of Ac-Hel$_1$-A$_5$-NH$_2$. For clarity, nitrogen atoms at backbone sites 3 and 6 are not shown. The minimization was initiated with backbone torsional angles of ϕ − 57°, ψ − 47°.

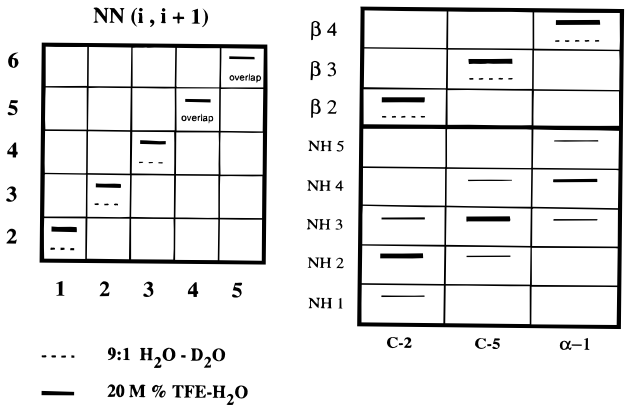

Figure 10. Summary of the helical NOE interactions observed for Ac-Hel$_1$-Ala$_6$-OH, in ROESY spectra shown in Figure 8a−d. Dotted lines signal an interaction observed in H_2O-D_2O (9:1); solid lines signal an interaction observed in 20 mol % TFE-d_4 80 mol % D_2O or in 20 mol % TFE-d_4 80 mol % (9:1) H_2O-D_2O (NN(i,i+1) only). For solid lines, the width of the line signifies relative intensity of the crosspeak.

cross peaks increase with the amount of TFE-d_3 added. ROESY spectra in 10 mol % TFE-d_3 were also taken (not shown) but were similar in intensity to the 20 mol % spectra.

Figure 8d shows a region of the ^{1}H phase-sensitive ROESY spectrum in 20 mol % TFE-d_3 −80 mol % (9:1 H_2O-D_2O) of Ac-Hel$_1$-344144-OH that allows comparison of the αN(i,i+2), αN(i,i+3), and αN(i,i+4) interactions between both template α-protons at C-2 and C-5 as well as the peptide α-proton at Ala-1. The detection at all three α-protons of αN(i,i+2) cross peaks along with strong αN(i,i+3) and weak but unambiguously detectable αN(i,i+4) cross peaks is uniquely consistent with a structural assignment of predominantly α-helical character.[29,34] The presence of a fraction of rapidly equilibrating 3_{10} helix cannot be excluded.

Figure 9 depicts the structure obtained from molecular mechanics simulations of a te state of an Ac-Hel$_1$-alanine helix containing six hydrogen bonding sites. Figure 10 summarizes the relative intensities of all observed NOE interactions. The ROESY spectra of the t state of Ac-Hel$_1$-A$_6$-OH in 20 mol % TFE−water reveal all the NOE interactions expected for an α-helix and no others. The ROESY spectra of the t state of Ac-Hel$_1$-A$_6$-OH in water alone reveal all helical NOE interactions that involve close van der Waals contacts, including the structure-defining αβ(i,i+3) interactions. As noted in Table 3, none of the medium- and long-range αN(i,i+j) interactions are likely to be detectable for a spectrum obtained from a frayed te state at low concentration under suboptimal NOE conditions. All NMR structural evidence is thus completely consistent with nucleation in aqueous solvents in the t state of Ac-Hel$_1$-A$_6$-OH of a helix that may be in rapid equilibrium with a small amount of 3_{10} structure but which is predominantly α, with a fractional population that is relatable to the measured template t/c.

Summary

The ^{1}H NMR properties of the unaggregated peptide-template conjugate Ac-Hel$_1$-A$_6$-OH in water have been shown to be insensitive to temperature, dilution, and addition of methanol, moderately sensitive to TFA, dilute salt, and urea, and dramatically sensitive to the helix-stabilizing alcohol TFE. The chemical shifts of the c state NH resonances of Ac-Hel$_1$-A$_6$-OH as well as their dependences on additives reflect a high degree of solvent exposure. By contrast, the chemical shifts and temperature dependences of te state resonances are solvent shielded, and the t state $^3J_{NH\alpha}$ values are helical in the central regions of the alanine peptide. CD spectra of the conjugate in water and TFE−water mixtures are consistent with t state helical character. For Ac-Hel$_1$-Ala$_6$-OH in water and in TFE−water mixtures, ROESY NOE interactions of ^{1}H NMR spectra are consistent with the presence of an α-helix. Analysis of the cs state resonances reveals no evidence for medium or long range structure. Comparison of properties of Ac-Hel$_1$-Ala$_6$-OH and the less constrained structural analog Ac-P-P-Ala$_6$-OH shows that the template Ac-Hel$_1$ acts at the N-terminus of the short alanine oligomer to initiate helical structure within the te state manifold.

Experimental Section

All Ac-Hel$_1$ derivatives were prepared, purified, characterized, and analyzed by ^{1}H NMR spectroscopy at 500 MHz as reported previously.[1,3] Coupling constants were obtained by direct measurement from the NH region of Ac-Hel$_1$-A$_6$-OH in H_2O-D_2O (9:1) at 25 °C; the estimated accuracy for the t-state resonances is ±0.2 cps, and for the slightly broader c-state resonances is ±0.3 cps. Assignments of NH resonances to specific amino acid residues of Ac-Hel$_1$-A$_6$-OH was carried out as described previously[1] and confirmed using the three selectively deuterated alanine derivatives used in the ROESY experiments.

Molecular modeling was carried out in vacuum without NOE constraints using the CHARMm QUANTA 3.3 software of Molecular Dynamics Inc as reported previously.[3] Molecular dynamics simulations were not employed, and the starting conformation for the modeling was assigned textbook α-helical or 3_{10}-helical values for ϕ and ψ.

Variable Temperature ^{1}H NMR Spectra. The spectra of Ac-Hel$_1$-A$_6$-OH in H_2O-D_2O (9:1), pH 1, 25 °C, were measured at 12 temperatures in the range of 5−65 °C using a Varian VTC4 temperature control apparatus. At each temperature the probe and sample were allowed to equilibrate for a minimum of 10 min before shimming followed by measurement. A methanol standard was used for calibration. NH chemical shifts showed no deviation from linear behavior, and slopes reported in Table 2 were determined by a linear regression.

^{1}H NMR Spectra in Urea, NaCl, TFA, and TFE Solutions. Chemical shift studies of Ac-Hel$_1$-A$_6$-OH (10.6 mM, 6.9 mg in 900 μL 9:1 H_2O-D_2O) in urea solutions (0−7.5 M) were carried out by adding solid urea directly to the NMR tube. The resulting volume was calibrated by comparison with an identical tube containing a known volume of pure water. The pH of the solution was monitored after each urea addition and maintained in the range of 1.76−1.86 by addition of small amounts of CF_3CO_2D or NaOD. At pH values <1, unacceptably large quantities of acid were required to control the pH.

Chemical shift studies of Ac-Hel$_1$-A$_6$-OH in NaCl solutions were carried out as described above, except that the pH was adjusted to 0.7 with TFA, and the salt concentration was adjusted by addition of aliquots of concentrated NaCl solutions. Chemical shift studies of Ac-Hel$_1$-A$_6$-OH in TFE solutions were carried out in a similar fashion. A solution (700 μL) containing Ac-Hel$_1$-A$_6$-OH in D_2O with TSP (pD *ca*. 1) was added to the NMR tube. Aliquots of TFE were injected directly into the NMR tube in a stepwise fashion and in the appropriate amount to raise the TFE mol percent value to the desired level.

Amide Hydrogen Exchange Studies. The Ac-Hel$_1$-A$_6$-OH used in exchange studies was lyophilized from H_2O after equilibration and dried in vacuum for >1 day. This sample was chilled under dry N_2 and dissolved in a D_2O solution containing sufficient TFA-d_1 to maintain its pH in the range of 3.2−3.6 in which the rate of exchange is minimal. Prior to addition of Ac-Hel$_1$-A$_6$-OH, the NMR tube containing this solution had been temperature equilibrated in the NMR probe, and the magnet shimmed. The solution in the cooled NMR tube was returned to the probe, and acquisition of FIDs was begun either immediately or after a very brief period of reshimming. Owing to time constraints, presaturation was not used to suppress the residual HDO signal. Each data acquisition usually consisted of 64 transients, requiring an acquisition time of ca. 2 min. The measured c state exchange rates for NH resonances at 10 °C for Ac-Hel$_1$-A$_6$-OH in D_2O, pD range of 3.2−3.6, are 0.018, 0.016, 0.020, 0.022, 0.044 min^{-1} for NH 1, 2, 3, 4, 5, and 6, respectively.

Circular Dichroism Measurements. All CD spectra were measured on an Aviv Model 62DS circular dichroism spectrometer. Dilution studies of Ac-Hel$_1$-A$_6$-OH by CD were conducted at room temperature by serial dilution of a sample of known concentration in 1, 10, and 20 mm cylindrical quartz cuvettes to a final concentration of 6.6 μM. Absorption of the perchlorate buffer used to maintain low sample pH prevented the use of longer cuvettes and thus further dilution.

CD spectra of Ac-Hel$_1$-A$_6$-OH in H_2O and in 20 mol % TFE−water were measured at 25 °C in a 1 mm strain-free quartz cell. A sample of Ac-Hel$_1$-A$_6$-OH was vacuum dried and dissolved in a 0.1 M perchlorate buffer, pH *ca*. 1, and carefully diluted to the appropriate concentration for measurement. An aliquot of this was combined with the appropriate amount of TFE for measurement of the TFE−water spectrum. The concentration of the Ac-Hel$_1$-A$_6$-OH solution was determined by sample hydrolysis followed by ninhydrin assay in an adaptation of the Rosen procedure[35] in which the observed optical density was calculated from ninhydrin analyses of standards containing known amounts of alanine. Control experiments established the accuracy of this procedure.

2D ^{1}H NMR Studies. In order to allow full relaxation and avoid distortions due to residual transverse magnetization, the proper 90° pulse width and the maximal T_1 values were measured for each 2-D experiment. Homospoil pulse consisting of a 90°−homospoil−90° sequence were also incorporated into the initial delay period for each experiment. For experiments in 9:1 H_2O-D_2O or its mixtures with TFE, the transmitter and decoupler were centered on the HDO peak, and the minimum level of decoupler power was used that provided adequate water suppression. Since nearly all conjugates were studied at pH ca 1.0, water suppression was achieved by decoupler presaturation for 1.5 n−2 s during the initial delay period of the pulse sequence. The intensity of the suppressed HDO peak was less than that of the template acetyl resonance. No symmetrization routines were applied to the 2-D spectra. ^{1}H−^{1}H DQCOSY and TOCSY spectroscopy experiments were used for peak correlations and to establish spin connectivities as reported previously.[1,3]

^{1}H−^{1}H ROESY Spectra. For the viscosities of the solutions studied and the substrate molecular weight range the magnitude of $\omega_o\tau_c$ causes NOE cross-relaxation processes to fall in the region between positive and negative enhancements. Accordingly, the commonly used NOESY 2-D NMR experiments failed to yield detectable NOE crosspeaks. The rotating frame 2-D NOE procedure (ROESY) effectively allowed detection of cross relaxation processes for these molecules. A t_o−90°−t_1−($\beta-\tau$)$_{16}$−t_2 pulse sequence was employed incorporating a mixing period of 300−350 ms during which a spin-lock field was applied. Mixing times <250 ms failed to provide adequate cross-relaxation, while mixing times >350 ms resulted in loss of signal intensity owing to T_1 relaxation. T_1 values were measured in the range of 0.1−1.5 s, and the t_o value was set in the range of 1.3−3.0 s. For experiments in HDO suppression of the water peak was achieved with presaturation throughout t_o, except for a 1 μs delay prior to the first sequence pulse. Generally the spin lock field was generated with a train of 16 30° (β) pulses resulting in a net field strength of 0.7−1.3 kHz. This time shared pulse technique,[36] combined with the low spin-lock field power,[37] minimized distortions due to Hartmann−Hahn magnetization transfer (HOHAHA) and coherence transfer (COSY) through scalar coupling. All analyses of spectra were carried out with attention to HOHAHA relay artifacts.[38] For spectra measured in D_2O the spectral window was expanded to twice the necessary span so that the transmitter frequency was placed downfield of the peak region to further minimize HOHAHA artifacts.[37] The spectra were acquired in the phase sensitive mode with quadrature detection using the hypercomplex phase cycling method.[39] The spectral widths varied from 4.5−5.5 KHz. Each data set, composed of 200−500 increments in t_1 with each FID in t_2 and digitized with 2K or 4K points, was expanded to 2K × 2K or 4K × 4K with zero filling and processed with a phase-shifted Gaussian weighting in both the F_1 and F_2 directions prior to Fourier transformation. After the Fourier transformation, a spline-fitted baseline correction was applied in each direction.

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