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Consistent Helicities from CD and Template t/c Data for N-Templated Polyalanines: Progress toward Resolution of the Alanine Helicity Problem

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Abstract: The helicity reporting parameters t/c and $[\theta]_{222}$ have been measured at 2, 25, and 60 °C in water for the solubilized polyalanine series Ac-Hel-A_n-^{t}LInp$_2$K$_4$W-NH$_2$ of length $4 \leq n \leq 14$ that bears the helix-initiating and monitoring N-cap Ac-Hel and the spaced solubilizer ^{t}LInp$_2$K$_4$W-NH$_2$ as a C-cap. Correlation of t/c with length shows that the helical propensity for $n \leq 6$ is ca. 1.0, consistent with our early reports, but that a dramatic increase in temperature dependence and helical propensity occurs for $n \geq 8$. A model based on hydrogen-bonding cooperativity is proposed to explain this finding, and both t/c and $[\theta]_{222}$ are modeled successfully by length-dependent alanine propensities at 2 °C of 1.03 for $n = 6$, 1.15, for $7 \leq n \leq 9$ and 1.26 for $n \geq 10$. The implications of these results for the energetics of helix formation by alanine-rich peptide sequences are discussed.

Polypeptides of appropriate structure assume helical conformations in water, and much recent attention has been directed toward accurate prediction of helicity from three structural features: the length of the peptide, its N- and C-capping functions, and its amino acid composition.[1] A key predictive parameter, the intrinsic helical propensity of alanine, remains controversial,[2] owing to disagreements concerning the validity of measuring techniques and appropriate peptide contexts. A recent breakthrough in our laboratory now permits study of water-solubilized, unaggregated, context-free polyalanines.[3] Demonstration of consistent results from independent helicity measuring techniques applied to these polyalanines now promises to resolve this long-standing problem. This is the first report of our new results.

Ten years ago we introduced the concept of a reporting helical conformational template, we demonstrated its feasibility in the form of a peptide N-cap Ac-Hel, and using NMR-derived t/c data we applied Ac-Hel to quantitative analyses of the helicity problem.[4,5] Doubt has recently been cast on the validity of the t/c analyses.[6] We now combine t/c and circular dichroism (CD) data to revalidate Ac-Hel as a peptide helicity monitor and use it to calibrate the alanine helical propensity for short polyalanine sequences.

Linked at the N-terminus of a peptide sequence, Ac-Hel induces helicity. The mechanism of this effect requires a brief analysis. In water a normally flexible, largely extended, disordered polypeptide molecule assumes a partially helical conformation if a local region contains a compact, ordered, helical structure of sufficient stability to offset loss of the high chain entropy of the normal disordered state. This enthalpic helix stabilization is usually attributed to a combination of internal hydrogen bonding, charge-dipole, and hydrophobic contributions. The stability of the helical region generally increases with its length, and this effect is expressed on a per residue basis as an equilibrium constant, the helical propensity, defined as the helix-joining tendency of an unordered amino acid sited at the terminus of a pre-existing helix.

Very short helical conformations containing three to six residues are too unstable to be detected directly, presumably because internal enthalpic stabilization is insufficient to compensate for loss of the conformational freedom of the peptide backbone. This entropic price is usually modeled as an initiation parameter. In the commonly used Lifson−Roig helicity algorithm,[7] the initiation parameter is expressed as v^2, and the relative stability of a helical conformation is modeled as the product of an initiation parameter v^2 and a series of w values that reflect the helix-stabilizing or destabilizing tendencies of the amino acids in the helical sequence.

Ac-Hel is a derivative of Ac-Pro-Pro that is conformationally constrained to adopt a preorganized helical peptide backbone.

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Two types of helical conformations can be envisaged for peptides that bear Ac-Hel as an N-cap. If the helical region fails to extend to the N-terminus, the conformational stability is low, because the helix initiation parameter remains v^2. If the helical region connects with Ac-Hel, the initiation parameter becomes $B \gg v^2$, and as a result the conformational stability is much higher. This is the mechanism of the helix-inducing effect of Ac-Hel.

The most commonly used measure of peptide helicity is based on an empirical linear correlation between CD residue molar ellipticity at 222 nm, $[\theta]_{222}$, and fractional helicity, FH, the fraction of potentially helical amino acid α-carbons that are actually part of helices.[8] Both the measured value of $[\theta]_{222}$ and the FH that is calculated from it are abundance-weighted averages over all peptide molecules. An unaggregated peptide in a strongly hydrogen-bonding solvent such as water forms a conformational manifold composed of a single completely helical conformation and many partially helical and nonhelical conformations.[7] The Lifson−Roig algorithm serves as a bookkeeper. For a potentially helical peptide of length n, it assigns a state sum of weights to 2^n of these conformations, from which mole fractions that define relative conformational abundances and other useful properties can be calculated.

Background

What is the t/c ratio and how is it used to monitor helicity? In addition to inducing helicity in a linked peptide,[4] Ac-Hel, sited at the N-terminus of a potentially helical peptide, allows measurement of helical stability[5] and detects conformational communication that exists throughout a helix.[9,10] The helicity-reporting properties of peptide conjugates of Ac-Hel result from analysis of their ^{1}H NMR spectra. In water, peptides N-capped by Ac-Hel exhibit two distinct sets of ^{1}H NMR resonances, corresponding to slowly equilibrating t and c rotamers of the acetamide function. The c-state leaves the linked peptide unstructured. Only the Ac-Hel t-state induces peptide helicity, which is proportional to the experimental t/c ratio, a simple linear function of the helical state sum.[5] Previously we have analyzed and reported t/c values for short polyalanines Ac-Hel-A_n-NH_2, but only up to their solubility limit of $n \leq 6$. We have also analyzed a t/c database obtained from 50 Ac-Hel-capped alanine-rich peptides of total length in the range 3−12 containing a single solubilizing Lys and have studied similar Arg, Orn, and His peptides.[10−12] The best fit to these data was obtained by assigning alanine a helical propensity only slightly greater than 1.0, in accord with previous reports from the Scheraga group,[13] but inconsistent with much larger values reported by the Baldwin[14] and Stellwagen[15] groups from CD studies of databases built largely from $(A_4K)_m$ oligomers. We now report

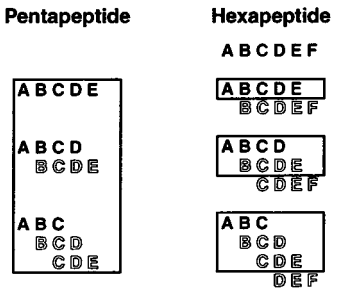

Figure 1. Major helical conformations for simple (solid and hollow lettering) and Ac-Hel-capped (solid) penta- and hexapeptide sequences. The $6 = (5 - 1)(5 - 2)/2 = (n - 1)(n - 2)/2$ conformations that are shared by both peptides are highlighted in boxes. The $(5 - 1) = 4 = (n - 1)$ new helical conformations of the hexapeptide are unhighlighted. Conformations that are selectively and strongly stabilized by the Ac-Hel cap are solid letters; only a single new solid conformation appears in the hexapeptide manifold.

both t/c and CD measurements for a more definitive series of Ac-Hel-capped unaggregated polyalanines, Ac-Hel-A_n-^{t}LInp_2K_4W-NH_2 with length $n \leq 14$. In these sequences the K_4W region solubilizes and provides a UV chromophore required for accurate measurements of $[\theta]_{222}$.

We have recently introduced a pair of ^{t}LInp_2 spacers (^{t}L = *tert*-leucine, Inp = isonipecotic acid) in WK_4^{t}LInp_2-A_n-^{t}LInp_2K_4-NH_2 polyalanine peptides and shown that the rigid extended conformation of the ^{t}LInp_2 region isolates the polyalanine core from the K_4 solubilizers.[3,16] The helical region of these spaced peptides is confined to the core; the spacers and solubilizing caps contribute insignificantly to the value of $[\theta]_{222}$.[3] Using t/c and CD data for a new solubilized series Ac-Hel-A_n-^{t}LInp_2K_4W-NH_2, we now reinterpret previous t/c data and derive new values for the helical propensity of alanine.

Analysis of Ac-Hel-peptide conjugates allows detection and characterization of helices formed by very short peptides, in the length range of three to eight residues. The properties of short helices have not been previously defined. This is an important gap in our knowledge since short helical conformations contribute significantly to the experimentally determined helicity of typical larger peptides.[7] Two fundamental problems arise if one attempts to fill this gap by CD data. Normally the mole fractions of unstable helical conformations formed by short peptides are very low and do not contribute significantly to an experimental $[\theta]_{222}$. Second, for helices formed by larger peptides that can be characterized by CD, the ellipticity signal is averaged over many substates that reflect the properties of both long and short helices. It is not possible to deconvolute such spectra to assign the contribution of each. These issues are central to the thrust of this report, and they are clarified in Figure 1, which shows the major helical conformations formed by two short peptides of sequences ABCDE and ABCDEF.

As noted in Figure 1, the total number of major helical conformations is $(n - 1)(n - 2)/2$, and as the peptide length n is increased by a single residue, all previous helical amino acid sequences are retained, but $(n - 1)$ new conformations are added. These span the range of lengths, from long to short. The added conformations thus contribute all helix lengths to the new manifold, and the resulting length-induced change in $[\theta]_{222}$ is an insensitive mirror of the relative stabilities of conformers containing long and short helical regions.

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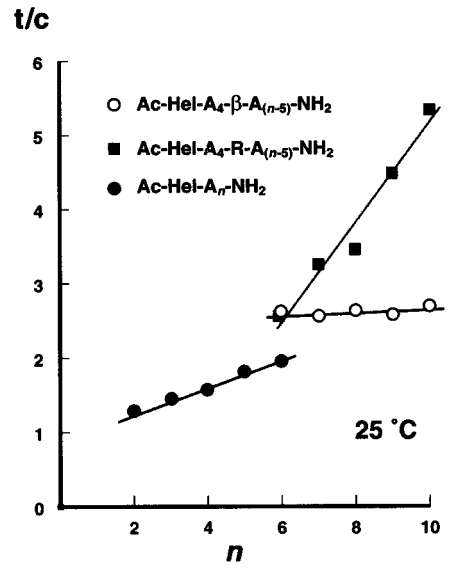

Figure 2. Plots of t/c vs length for three peptide series measured in water at 25 °C. (●) Ac-Hel-A_n-NH_2, $2 \le n \le 6$;[5,10] (■) Ac-Hel-A_4-R-$A_{(n-5)}$-NH_2;[8] (○) Ac-Hel-A_4-β-$A_{(n-5)}$-NH_2,[8] $\beta = \beta$-aminoalanine. For peptides in the size range of this study, t/c is proportional to a sum of weights for the Ac-Hel-stabilized helical conformations, all of which are initiated at the N-terminus.

Owing to the helix-inducing property of Ac-Hel, one can for the first time detect helicity for peptides of very short lengths, but there is a second reason Ac-Hel peptides are unique tools for detecting differences in the properties of short and long helical peptide conformations. As seen in Figure 1, the conformational manifolds of peptides bearing strongly helix-stabilizing caps differ from those belonging to simple peptides.[5] The presence of the N-cap dramatically and selectively increases the relative abundances of conformations, solid, in which the helical region starts at the peptide N-terminus. Extension of the chain by one residue adds only one new solid conformation, which is longer than any of its precursors. No cap-stabilized short conformations are added, the number of cap-stabilized solid conformations grows directly with length n, not with $n^2/2$, and the conformational average of the dominant gray conformations is highly helical at the N-terminus and frays progressively toward the C-terminus. The amino acid residue at that terminus participates only in the completely helical conformer. If w is greater than 1.0, this conformer must be more stable and thus more abundant than any of its precursors. For these reasons, any large length-dependent change in helical stability should be revealed in a plot of t/c vs n for an Ac-Hel-capped homopeptide length series.

This point is demonstrated by Figure 2, which allows comparison of three sets of our previously reported t/c data.[10,12] For the homopeptide series a plot of t/c vs n is expected to show an abrupt increase at a particular value of n only if the length increase introduces new, unusually stable conformers. If the stabilities of new conformers are similar to those of its precursors, the new t/c data points should be linearly correlated with earlier points on the graph. Exactly this behavior is seen in Figure 2 when the tetrapeptide sequence of Ac-Hel-A_4-NH_2 is extended by one or two alanine residues. The linearity of this t/c plot for a series that starts at the Ac-Hel junction is consistent with an alanine helical propensity w of 1.0 ± 0.05.[5,10]

Very different behavior is seen if either an Arg or a β-aminoalanine (β) is introduced toward the C-terminus of the alanine sequence.[10] The t/c value increases substantially, implying that new added conformational states are more strongly stabilized than their precursors, consistent with the documentation of large helix-stabilizing effects of C-terminally sited positively charged amino acids.[17]

Further extension of the Arg and β-series by addition of longer C-terminal polyAla sequences differentiates these two cases. For the β peptide series, t/c shows no detectable effect of chain extension. Since the t/c reporter function is sited at the chain N-terminus, this result implies that in the β-series, new helical conformations that may appear within the C-terminal A_n region cannot partake of the initiating effect of the Ac-Hel function and are isolated from it by the β residue. A helix spontaneously initiated to the right of β fails to communicate with the reporter function of Ac-Hel, and β acts as a strong helix-stabilizing stop signal.

Replacement of β by Arg results in length-dependent stabilization for all Arg-containing helical conformations; a change in stabilization at the C-terminus by chain elongation is clearly reflected by the t/c reporter at the N-terminus. Similar behavior is seen for series of peptides Ac-Hel-A_4-X-A_n-NH_2 for which the Arg residue is replaced by Lys, Orn, or His.[10−12] The slope of the second part of the plot decreases significantly in the order Arg > Lys, Orn > His, consistent with relative helix propensities documented by others.[18]

We also characterized the temperature dependence of t/c for the series Ac-Hel-A_5-K-A_n-NH_2.[12] Unlike the short Ac-Hel-capped polyalanines, these alanine-rich mono-Lys peptides show strong melting behavior. We previously modeled these data accurately by attributing temperature dependence to the lysine residue.

Qualitative Properties of Ac-Hel-Capped Polyalanines

Prior to this work, our database of t/c properties for Ac-Hel-linked polyalanines was confined for solubility reasons to the short series Ac-Hel-A_n-NH_2, $n \le 6$, of Figure 2. The t/c properties of this series are shown in greater detail in Figure 3. Normal melting behavior has been documented for simple peptides of length greater than ca. 15 residues,[19] but no decrease in t/c is detectable with increase of temperature for any member of the short N-capped peptides.[20] CD spectra exhibited by these short conjugates are weak, but if appropriate corrections for the Ac-Hel contributions are applied to TFE titration spectra for the series $3 \le n \le 6$, deconvolution based on t/c-derived mole fractions allows calculation of CD spectra for the limiting nonhelical (cs + ts) and helical (te) states for the penta- and hexapeptides.[21] These correspond to literature reports for respective helical and nonhelical conformations.[22]

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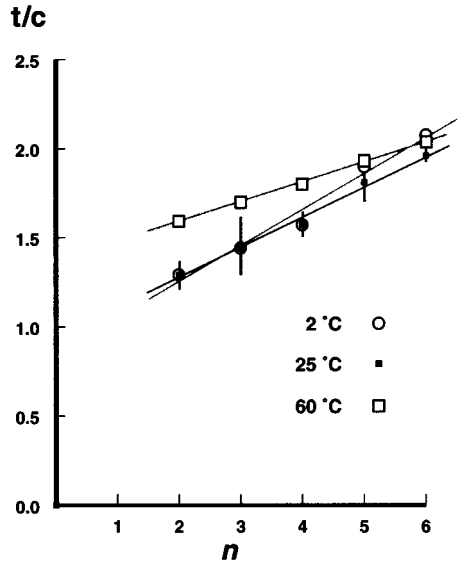

Figure 3. Temperature dependence of t/c data for the series Ac-Hel-A_n-NH_2 in water. t/c values (not shown) at 25 °C for Ac-Hel-A_n-^{t}LInp$_2$K$_4$W-NH_2, $n = 4$ and 6, were consistent within experimental error with the above series. Error bars for 25 °C data are standard deviations for three measurements.

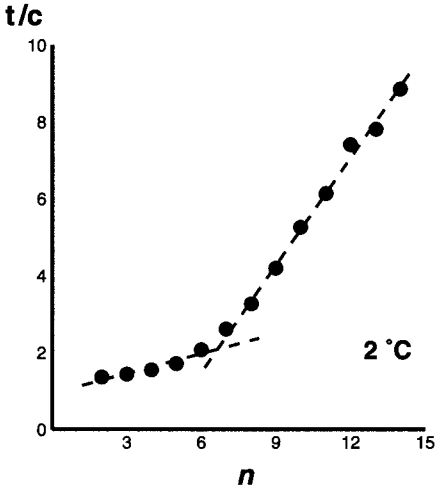

Figure 4. Length plot of t/c data for the series in water at 2 °C for Ac-Hel-A_n-^{t}LInp$_2$K$_4$W-NH_2, $2 \leq n \leq 14$. Data for $2 \leq n \leq 6$ are averaged with data for the series Ac-Hel-A_n-NH_2 of Figure 2. Dotted lines show linear regressions for data in the two ranges $2 \leq n \leq 6$ and $8 \leq n \leq 14$, demonstrating a dramatic slope increase in the transition region $6 \leq n \leq 8$. As seen in Figure 5, a qualitatively similar plot is seen at 25 °C.

Despite their anomalous melting and CD properties, by other criteria these peptides appear to form unexceptional helices. As noted above, they exhibit conformational communication, responding as a conformational unit to site modifications. The expected increases in t/c are induced by TFE[5] and perchlorate,[23] and the NOE interactions characteristic of α-helices are present.[4]

When t/c data for a more complete Ac-Hel-capped polyalanine series, Ac-Hel-A_n-^{t}LInp$_2$K$_4$W-NH_2, $4 \leq n \leq 14$, are examined, Figure 4, an abrupt increase in helical stability is apparent when the length of the peptide region is extended from six to eight residues. Data points within the region $2 \leq n \leq 6$ and the region $7 \leq n \leq 14$ give excellent independent linear correlations. Remarkably, if the plot of Figure 2 for the Ac-

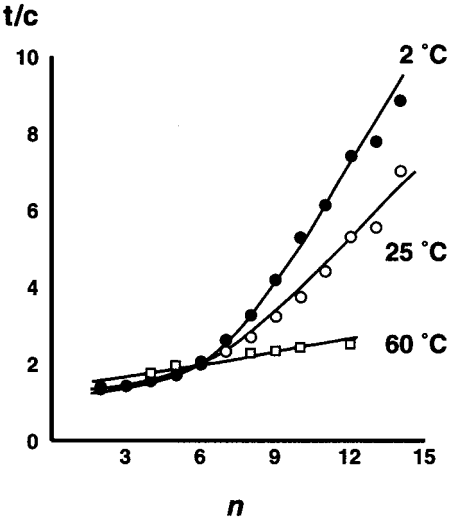

Figure 5. Temperature dependencies of t/c values for the series Ac-Hel-A_n-^{t}LInp$_2$K$_4$W-NH_2, water, 2 °C (●), 25 °C (○), and 60 °C (□), graphed as functions of polyalanine length *n*. Connecting curves are calculated from the two-parameter Lifson−Roig (L−R) fit described in the text and Experimental Section.

Hel-A_4-R-$A_{(n-5)}$-NH_2 series is superimposed on the 25 °C data of Figure 5, for $n > 6$, t/c data for the homoalanine and mono-Arg series are found to be similar. The large increase in slope observed for the longer peptides must therefore be attributed primarily to their length and not to the presence of a basic amino acid residue. This conclusion calls for a reinterpretation of all our previous t/c data obtained for N-capped Lys-, Arg-, and His-containing polyalanines. The helical propensity of alanine itself must be length dependent. It shows a significant increase that is likely to begin for peptide conformers with lengths as short as five residues but cannot become fully operative until the conformational manifold contains helical conformations of eight residues or more.

We focus here on defining the helical propensities of alanine quantitatively for polyalanine peptides. We leave the more complicated issue of defining the helix-stabilizing roles of Arg, Lys, Orn, and His to a later report but offer two preliminary but firm empirical generalizations. Within Ac-Hel-initiated alanine-rich helices, the presence of single amino acid residues bearing positively charged side chains is not the primary determinant of helicity, but it does strongly modulate the underlying energetics of alanine helices and shifts the relative stabilities of conformers within the manifold. Single Arg, Lys, or Orn residues that appear within the center or C-terminal regions of an Ac-Hel-capped alanine helix are characterized by larger mean helical propensities than those of alanine itself. In this context these residues are helix stabilizing.

Quantitative Modeling of t/c Data for the Series Ac-Hel-A_n-^{t}LInp$_2$K$_4$W-NH_2 ($4 \leq n \leq 14$): A Hydrogen-Bonding Cooperativity Model for Helix Formation

What precedents and plausible models exist for this significant length-dependent change in helical energetics, which almost certainly must reflect a corresponding structural change? In globular proteins α-helices exhibit large and characteristic structural variations,[24] and recent calculations suggest that these

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are correlated with changes in hydrogen-bonding geometry and energetics.[25] Within the X-ray structural database, the helical dihedral ϕ angle of the protein backbone can vary over the range $-45°$ to $-85°$ without change in α-helix hydrogen-bonding connectivity, provided the associated ψ angle varies in a compensatory manner ($\psi \approx -107° - \phi$).[24] The tilt of helix amide planes relative to the helix axis depends on ϕ, as does the linearity and orientation of the amide−amide hydrogen bonds. A change in the strength of these H-bonds could account for our length-dependent t/c findings.

Theory predicts an increase in the average strength of the amide−amide H-bond in water when chains of intramolecularly H-bonded secondary amides are lengthened.[26] We invoke this H-bonding cooperativity effect as the most likely explanation for the increased stability of alanine helical conformations observed for Ac-Hel-capped polyalanines of length greater than seven residues. Hydrated helical conformations in which the amide carbonyl groups are tilted outward toward the solvent have been characterized within globular protein structures.[27] Abnormally weak amide−amide H-bonds are expected to appear in these helices, and theory predicts that they should show unusually weak CD signatures.[8] They may correspond to our very short helical conformations.

We have defined a Lifson−Roig-derived computation model that incorporates a parameter that reflects H-bonding cooperativity. An α-helix contains three independent chains of continuously H-bonded amides. For simple N- and C-capped peptides of length $5 + 3i$, these chains are of equal length and contain $(i + 1)$ amide−amide H-bonds. The energetic effect of adding a new amide−amide H-bond is expected to be largest for small i and to converge to a limit as i increases. A model can be envisaged in which a separate energetic parameter is used for each i, and three such parameters are combined for the length of each helical conformer in the manifold. We have briefly explored such models,[28] but for our particular peptide length series they prove to be computationally equivalent to the simple single-parameter model developed below.

As noted in the Introduction, a simple Lifson−Roig (LR) model for calculating experimental helicity parameters of a homopeptide series relies on two parameters, a helix initiation constant v, which we assign a literature value of 0.048,[29] and a helical propensity w. Calculation of t/c values from L−R-derived state sums requires knowledge of two additional parameters: an additive parameter A, which defines the intrinsic tendency of the Ac-Hel cap to assume t or c states, and a multiplicative parameter B, which is the helix initiation constant for peptide helices linked to Ac-Hel through an alanine residue.[4] The value of A is fixed by the Ac-Hel function; the value of B depends on local interactions between the first few residues of the helix and the Pro-Pro elements of the template. Both parameters are thus defined by the first five residues of the Ac-Hel polyalanine series of Figure 4. From studies of a wide range of peptides and analogues, A and B are characterized as temperature independent in water, 0−30 °C, as $A = 0.80-0.85$ and $B = 0.15-0.20$.[4,12] In accord with previous modeling of this short series we assign $w = 1.0$ for $n \leq 4$.[4,12]

We model H-bonding cooperativity as fully operative for all three H-bonded amide chains of peptides of length $n \geq 7$, and we assign a new free parameter w' for all conformations that meet this length condition. To allow a smooth mathematical transition, helical propensities for conformations of length 5 were set at $w'^{(1/3)}$, and those of length 6 at $w'^{(2/3)}$. The value of the template constant A was set equal to 0.79, but B was treated as a free parameter. Thus, for the 2 °C temperature series, we used a pair of free parameters, B and w', and additional pairs for the 25 and 60 °C series.

The best least-squares fit of the L−R modeling of the 13-member data set at 2 °C gives $w' = 1.29$, $B = 0.177$; the corresponding fit at 25 °C gives $w' = 1.215$, $B = 0.176$. At 60 °C the quality of data and the range of its variation are not optimal; w' is consequently less well defined but lies the range $1.01 < w' < 1.06$. It is noteworthy that the values of B obtained at 2 and 25 °C are identical and consistent with our earlier assignments, as listed above.[4,12]

Experimental t/c data points at the three temperatures of this study are shown in Figure 5. Curves were calculated from B and w' using a L−R algorithm. These faithfully mirror all details of the experimental t/c data. Under the assumptions of the model and in water at the temperatures of the study, these template constants and w' values define all helical properties of conformational substates and of the polyalanines themselves: the substate mole fractions, their overall fractional helicities, and their site helicities.

$$[\theta]_{222} \approx [\theta_n]_{222}\mathrm{FH} \tag{1}$$

$$[\theta_n]_{222} = [\theta_\infty]_{222}(1 - x/n) \tag{2}$$

where

n is the length of the potentially helical region

$[\theta_n]_{222}$ is the residue ellipticity of an idealized 100% helical peptide of length n

$[\theta_\infty]_{222}$ defines the limit of function (3) for very large n

x corrects for end effects

As noted in the Introduction, fractional helicity is usually taken as proportional to the experimental $[\theta_\infty]_{222}$ as seen in (1), with a length-dependent proportionality constant $[\theta_n]_{222}$ that can be calculated from a two-parameter equation, (2). It should thus be a simple matter to test the compatibility of $[\theta]_{222}$ and t/c data. Unfortunately, for alanine-rich helices there are currently several plausible choices for these parameters, and the problem presented by these choices is examined in a later section. Before addressing it we present results of a new and more general test of the mutual consistency of t/c and $[\theta]_{222}$ data.

(25) Mahadevan, J.; Lee, K.-H.; Kuczera, K. *J. Phys. Chem. B* **2001**, *105*, 1863−1876.
(26) Mehler, E. L. *J. Am. Chem. Soc.* **1980**, *102*, 4051−4056. Sheridan, R. P.; Lee, R. H.; Peters, N.; Allen, L. C. *Biopolymers* **1979**, *18*, 2451−2458. Guo, H.; Karplus, M. *J. Phys. Chem.* **1994**, *98*, 7104−7105.
(27) Blundell, T.; Barlow, D.; Borkakoti, N.; Thornton, J. *Nature* **1983**, *306*, 281−283.
(28) Helical conformations in the length range of 5−14 residues contain three different H-bond chain lengths, each of which must be assigned a separate weight in a model that rigorously distinguishes H-bonding cooperativity effects. Theory provides limited guidance concerning their relative magnitudes, but a monotonic convergence to a limiting energetic effect per H-bond is expected as n is increased. Many functional choices are possible, and the data at hand cannot be used to distinguish them. Modeling shows that all likely models yield data fits that are comparable to the fit observed with a single parameter but are only slightly superior to it. This finding reflects the averaging of weights for individual conformations that occur when t/c values are assigned to a helical manifold.
(29) Rohl, C. A.; Scholtz, J. M.; York, E. J.; Stewart, J. M.; Baldwin, R. L. *Biochemistry* **1992**, *31*, 1263−1269.

Comparisons of t/c and CD Data for the Ac-Hel-A_n-^{t}LInp$_2$K$_4$W-NH$_2$ Series: Validation of Ac-Hel as a Quantitative Helicity Reporter

We need to show that even in the absence of accurate values for the x and $[\theta_\infty]_{222}$ ellipticity parameters of (2), the experimental t/c and $[\theta]_{222}$ values correlate quantitatively. A linear relationship between them can be argued to provide the most rigorous proof that two experimental quantities correlate; it is subject to a refined statistical test, and its quality can be validated by simple visual inspection.

Inspection of the data of Figure 5 shows that a poor linear correlation exists between t/c and the single variables length n and temperature; a good correlation is not expected since t/c is clearly a complex function of both variables. The collective t/c values of Figure 5 also do not linearly correlate with experimental $[\theta]_{222}$ values measured under corresponding conditions, or with FH values that might be calculated from them. We conclude that an accidental linear correlation is unlikely if t/c values are paired with $[\theta]_{222}$ values that have been transformed by an arbitrary function.

Since t/c is the parameter at issue, in seeking correlations we have chosen not to transform its values by any mathematical operations. We use modeling-derived data to show that the transformation $[\theta]_{222} \longrightarrow [\theta]_{222}/([\theta_{test}]_{222} - [\theta]_{222})$ yields a function of $[\theta]_{222}$ with the same range as t/c, as noted in the Appendix. This function correlates linearly with t/c only if both t/c and $[\theta]_{222}$ model intrinsic helicity for the Ac-Hel series.

The scope of this correlation is greatly extended by two of its other properties that are demonstrated in the Appendix. First, if one varies the controversial parameter $[\theta_\infty]_{222}$ of (2), replacing it by $[\theta_{test}]_{222}$, very large deviations of $[\theta_{test}]_{222}$ from $[\theta_\infty]_{222}$ do change the slope substantially but do not affect the linearity of the correlation. Second, the quality of the correlation is not affected if pairs of t/c and $[\theta]_{222}/([\theta_{test}]_{222} - [\theta]_{222})$ values calculated for different peptide lengths and w' values are included.

As seen in Figure 6, within experimental error the quality of our data correlation for all t/c and transformed $[\theta]_{222}$ data points is comparable to that seen for Figure 9 of the Appendix, which is constructed from modeled data points. We conclude that for the polyalanine series of this study, CD and t/c data indeed provide independent, complementary measures of peptide helicity.

As detailed below, the appropriate value of $[\theta_\infty]_{222}$ for the alanine-rich helices of this study is currently under refinement. It is therefore premature to comment on the similar slopes seen in plots of Figures 6 and 9. We do note that in the future it is likely that such comparisons will allow ellipticity functions (2) to be calibrated for classes of helices formed by peptides containing particular amino acid compositions or sequences. Current use of (1) and (2) to calculate FH from $[\theta]_{222}$ data rests on an untested assumption that changes in amino acid side chains have no effect on $[\theta_\infty]_{222}$, which is believed to depend only on peptide backbone conformation. Slopes of plots such as Figure 6 may in the future permit quantitative tests of the errors that may be implicit in this assumption.

Correlation of CD Data for the Ac-Hel-A_n-^{t}LInp$_2$K$_4$W-NH$_2$ Series with t/c-Derived w' and B Values

What is the nature of the current $[\theta_\infty]_{222}$ controversy? There is little or no uncertainty concerning the appropriate value of

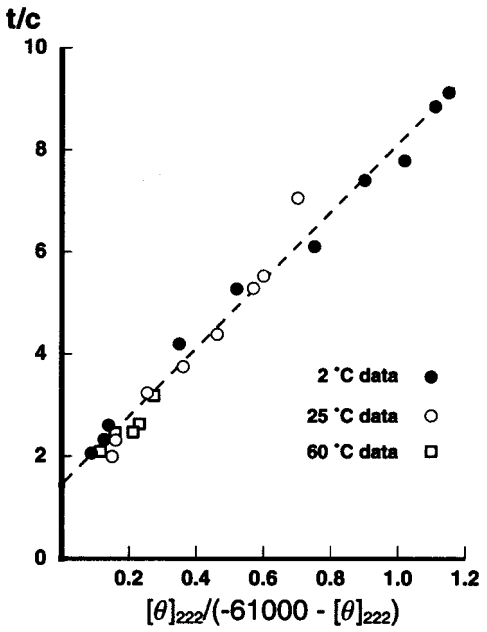

Figure 6. Comparison of the t/c data points shown in Figure 5 with corresponding values of $[\theta]_{222}/([\theta_{test}]_{222} - [\theta]_{222})$, calculated from experimental $[\theta]_{222}$ values measured in water for the series Ac-Hel-A_n-^{t}LInp$_2$K$_4$W-NH$_2$, and with $[\theta_{test}]_{222} = -61\,000$. The correlation coefficient is 0.98. Recalculation using $[\theta_{test}]_{222} = -43\,000$ gives a comparable linear fit. (See Figure 11 in the Appendix.)

$[\theta_\infty]_{222}$ when modeling helices found in globular proteins or in helix-coiled coils of the tropomyosin or leucine zipper classes. For these cases values for FH are defined as close to 1.0 by rigorous evidence, and the recently proposed value $[\theta_\infty]_{222} = -37\,000$ is likely to be correct.[24] The controversy hinges on the question of whether universal values of these parameters accurately describe the behavior of all helical peptides, irrespective of their contexts and amino acid composition. In particular, the proper value of $[\theta_\infty]_{222}$ for completely hydrated polyalanines or alanine-rich helices in water is much less well defined. With these peptides, no length calibration for (2) exists that is based on independent rigorous characterization of FH. Recently we have presented evidence that the most frequently cited parameters,[14,30] $[\theta_\infty]_{222} = -43\,000$ and $x = 2.5$ for (2), which allow calculation of the limiting value of $[\theta_n]_{222}$, the per residue ellipticity for a hypothetical 100% helical peptide of length n, grossly underestimate experimental ellipticities for the N-capped Baldwin−Marqusee peptide Ac-Hel-$(A_4K)_4A_2$-NH$_2$ and its analogues at 2 °C in water.[31] Although this peptide cannot approach 100% helical character, we observed $[\theta]_{222} = -51\,000$, or 30% greater than the limiting value calculated from (1) and (2) using the above-cited parameters. Clearly, −43 000 is a gross underestimate of the appropriate value of $[\theta_\infty]_{222}$ for these peptides. The same point can be made by calculating bounds for $[\theta_n]_{222}$ from the largest $-[\theta]_{222}$ values observed for the polyalanine series of the present study. The w values derived from t/c analysis set the fractional helicity at 2 °C for the Ala_{14} peptide as 0.68. For a 14-residue peptide, $-[\theta_{14}]_{222}$ is calculated as 35 400 if $-[\theta_\infty]_{222} = 43\,000$. Multiplying by 0.68 yields −24 000 for calculated $[\theta]_{222}$, which is 26% smaller than the experimental value. Nor is this kind of inconsistency unique to

(30) Luo, P.; Baldwin, R. L. *Biochemistry* **1997**, *36*, 8413−8421.
(31) Wallimannn, P.; Kennedy, R. J.; Kemp, D. S. *Angew. Chem., Int. Ed.* **1999**, *38*, 1290−1292.

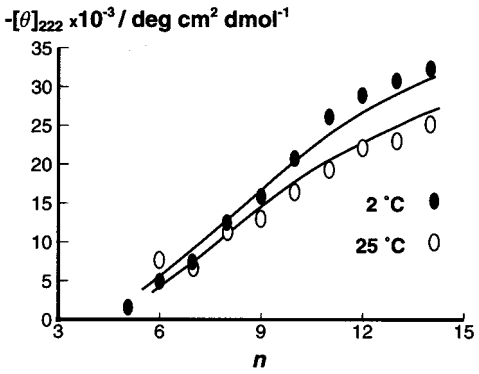

Figure 7. Plot of template and end-cap-corrected $-[\theta]_{222}$ data points measured in water vs length of alanine region for Ac-Hel-A_n-^{t}LInp$_2$K$_4$W)-NH$_2$. Curves were calculated using B and w' parameters derived from t/c data and the ellipticity function described in the text, applied to a L−R algorithm.

Ac-Hel-capped peptides. We have recently measured $[\theta]_{222}$ values at 2 °C for a spaced, solubilized series of simple polyalanines of length ranging from 12 to 45 residues.[16] Experimental values of $[\theta]_{222}$ for the longer of these C- and N-terminally frayed polyalanine helices lie in the range of −42 000 to −44 000, with FH < 1.0, implausibly close to $[\theta_\infty]_{222}$ at −43 000.

Our report of Ac-Hel-capped Baldwin−Marqusee peptides included a function for $[\theta_n]_{222}$ that, when used in a L−R analysis, approximated experimental $[\theta]_{222}$ values satisfactorily.[31] For helices of length <10, we set x in (1) to 2.5 and $[\theta_\infty]_{222}$ to −43 000 in accord with common usage, but we replaced the $[\theta_\infty]_{222}$ value of −43 000 [30] by −61 000 for longer helices. We adopt a slightly modified form of this function for the present study. To add precision to $[\theta_n]_{222}$ for shorter peptides, $[\theta_8]_{222}$ was characterized as −36 000 for $n = 8$ through protection factor[32] measurements on the exceptionally helical[33] N- and C-capped Ala_8 peptide Ac-$^\beta$D-Hel-A_8-β-InpK$_2$W-NH$_2$.[34] Using this value, setting $n = 8$, $x = 2.5$ in the functional form $[\theta_\infty]_{222}(1 - x/n)$, and solving for $[\theta_\infty]_{222} = -53\,000$ defines our ellipticity function for shorter peptides, $n \leq 9$, as $-53\,000(1 - 2.5/n)$ and $-61\,000(1 - 2.5/n)$ for $n > 9$.

For the Ala_n series at 2 and 25 °C, Figure 7 plots length dependence of the experimental $-[\theta]_{222}$ data. Using the w' and B calculated from t/c data and the above $[\theta_n]_{222}$ function, a L−R analysis yields the 2 and 25 °C curves shown in the figure. The calculated curves reproduce the sigmoid features of the length dependence of the $[\theta]_{222}$ data, and differences between calculated and experimental ellipticities lie within the error of measurement.

We conclude that a two-parameter cooperative H-bonding L−R model combined with a plausible ellipticity function that uses separate $[\theta_\infty]_{222}$ for short and long helical conformations successfully models both t/c and $[\theta]_{222}$.[35] For this system, t/c and $[\theta]_{222}$ provide equivalent helical characterization, although only the t/c data uniquely permit characterization of the helical behavior of short helical conformations.

Summary and Consequences

In this report we have introduced new t/c data for an extended Ac-Hel-A_n series that addresses the concerns previously raised by our critics,[36] and we have introduced a new correlation between t/c and $[\theta]_{222}$ that establishes t/c as a helicity reporter with unique capacity for characterizing the energetics of helix formation by very short peptides. In the course of this study, we were led to the novel conclusion that different helical propensities are characteristic of short and long polyalanine helices.

What is the practical significance of our dual w values? Since the contribution of short helical conformations is maximal for very short helices and relatively insignificant for helical conformations of length 14 at 2 °C, we cannot approximate overall w values for our length series as a simple average of w and w'. A good approximation to the desired mole fraction-weighted average $\langle w \rangle$ is obtained by carrying out a normal noncooperative L−R modeling for each peptide length. Thus, for the 2 °C t/c series, for $n < 7$, $\langle w \rangle = 1.03$, approximating the alanine propensities previously published by our group[5] and by Scheraga's group.[2a,11] For $6 < n < 9$, $\langle w \rangle = 1.15$; for $n \geq 10$, $\langle w \rangle = 1.26$.

Figure 5 shows that we have previously underestimated the complexity of the helix-stabilizing role of alanine in short alanine-rich peptides and that we have incorrectly assigned the helical melting properties of alanine-rich lysine conjugates primarily to temperature-dependent changes in the helical propensity of lysine.[11,12] We feel that until more complete data are available it is premature to generalize our w values to systems other than polyalanine. Their relevance to peptides and proteins of biological origin in which isolated alanine residues appear within other amino acid contexts is yet to be established, but that proof is currently in process. Preliminary results suggest that helical propensities for guest amino acids that have been measured in our polyalanine contexts will be found to correlate well with recent studies of the stability of analogous site mutants in helical proteins.[37] We do draw attention to the excellent match between the length range of this study and the average length of helices found in water-soluble globular proteins that have been characterized by X-ray structural analysis.

More work remains before the helical polyalanine context can be said to be understood. In an independent study, we have successfully tested the generality of our cooperativity model by applying it to the solubilized simple polyalanine series of length 12−45 residues.[16] Further assessments of its correctness and scope await a rigorous experimental characterization of the ellipticity function and a clearer understanding of its possible

(32) Englander, S. W.; Kallenbach, N. R. *Q. Rev. Biophys.* **1984** *16*, 521−655.
(33) Maison, W.; Arce, E.; Renold, P.; Kennedy, R. J.; Kemp, D. S. *J. Am. Chem. Soc.*, **2001**, *123*, 10245−10254.
(34) For the peptide Ac-$^\beta$D-Hel-A_8-^{t}L-Inp$_2$K$_4$W-NH$_2$ at 2 °C in water, $[\theta]_{222}$ is −34 000, which when divided by the fractional helicity of 0.94 yields $[\theta_8]_{222} = -36\,000$. The fractional helicity was calculated as the average of the reciprocals of the protection factors for the peptide alanine NH groups, calculated as the rate constants for NH → ND exchange in D_2O, pH 4, relative to those for the nonhelical peptide Ac-A_3-OH.
(35) As seen in Figure 6, $[\theta]_{222}$ data at 60 °C show an expected very small variation. Although satisfactorily modeled from t/c-derived helicity parameters, they provide no revealing test of curve shape correlation and are not included in Figure 7.
(36) Significant issues raised by our critics were the presence of a Lys residue in our previously reported peptides that might cause unknown bias in t/c ratios, and the absence of t/c temperature dependence for short alanine helices. The above analyses address both these issues. Rohl and Baldwin[6] also attempted a fit to our data in which the template constants A and B were treated as free parameters, which implies that they must vary significantly with peptide length, even when average helical lengths isolate the template region from plausible chemical influence of the helix C-terminus. As noted above, we believe A and B are local constants and draw attention to the quality of fit to data that is provided by our current model.
(37) Myers, J. K.; Pace, C. N.; Scholtz, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2833−2837.

context dependence, independent falsification tests of our cooperativity hypothesis, confirmation that our polyalanine-derived helical propensities also apply to alanines found within normal peptide sequences, and clarification of the complex roles played by charged amino acids within helical peptides. These studies are currently in progress.

Experimental Section

Peptides were synthesized on a Pioneer peptide synthesizer using Fmoc solid-phase peptide synthesis as described previously.[33] Purification and characterization were carried out on a Waters 600 HPLC with a 996 detector using YMC C18 ODS-AMQ columns.[3] A characterization table of mass spectral data obtained on a Waters ZMD mass spectrometer can be found in the Supporting Information. Circular dichroism measurements were obtained on an Aviv 62DS circular dichroism spectrometer.[33] Peptide concentration was determined on a Cary 300 UV−vis spectrometer utilizing the Trp chromophore (ϵ_{280} = 5560 cm^{-1} M^{-1}).[16] A sample CD spectrum and details concerning the cap correction can be found in the Supporting Information along with analytical ultracentrifuge, t/c, and NHND exchange results.

Lifson−Roig (L−R) Algorithms for Fits to t/c Data (Figure 5), CD Data (Figure 7), and All Calculations in the Appendix. Rather than conventional 3 × 3 or 4 × 4 matrixes, these calculations use the 8 × 8 matrixes that we have introduced previously.[16,31] All these give equivalent polynomial state sums, with two major differences. First, the state weight for a helical conformation of length k is t^2w^k, and second, in the initial state sum calculation, a conventional v weighting is used for isolated helical conformations, but a t^2 weight is used for helices. At the evaluation stage of the calculation, we set $t = v = 0.048$, according to common usage.[29] The double t,v weighting system for helix initiation allows easy partitioning of the state sum; for example, χ_{Hel-k}, the mole fractions of conformations containing uninterrupted helices of length k, is (1/SS)(sum of all polynomial terms containing t^2w^k as a coefficient). The partitioning of state sums into such terms is rapid and efficient with modern PC-based computational algorithms.

The ratio t/c is modeled as in (3), and the t/c ratio intrinsic to the template Ac-Hel, ts/cs, is set equal to a peptide-invariant parameter A = 0.76, as previously modeled.[5] The template initiation parameter B must be multiplied by the ratio SS_{te}/SS_{cs} of L−R peptide state sums.

$$t/c = (ts + te)/cs = ts/cs + te/cs = A + B(SS_{te}/SS_{cs}) \qquad (3)$$

The state sum SS_{cs} is a normal peptide helical state sum, modified slightly from that reported previously,[31] $aa{\cdot}m^{(n-1)}{\cdot}mc{\cdot}ab$, where aa = (0,0,0,0,0,0,1,1), ab = (0,1,0,1,0,1,0,1), and m is the matrix with nonzero elements as follows: (1,1) = w; (1,2) = tw; (2,3) = 1; (2,4) = 1; (3,5) = tw; (3,6) = v; (4,7) = 1; (4,8) = 1; (5,1) = w; (5,2) = $v^2/(tw)$; (2,3) = 1; (2,4) = 1; (3,5) = tw; (3,6) = v; (4,7) = 1; (4,8) = 1. Matrix mc introduces a capping parameter for the ^{t}L residue that terminates the polyalanine sequence. As calculated from data reported elsewhere,[38] c = 0.77 for measurements at 2 °C, and c = 1.0 for measurements at 25 °C. With the exception of the term (1,2) = twc, all terms of mc equal the corresponding terms of m.

The te state sum SS_{te} is calculated from the matrix product $aa{\cdot}n{\cdot}nn{\cdot}m^{(n-3)}{\cdot}mc{\cdot}ab$, where the nonzero coefficients of n are (7,5) = w; (7,6) = w; (8,7) = 1 = (8,8), and the nonzero coefficients of nn are (5,1) = w/t; (5,2) = w; (6,3) = 1 = (6,4), (7,5) = tw; (7,6) = v; (8,7) = 1 = (8,8). These coefficients were determined by the weightings of helical substates for each peptide length n. As an example, the 16 weightings for n = 4 are as follows: hhhh, w^4 c; hhhc, w^3; chhh, t^2w^3c; hhch, w^2v; hchh, wv^2; hhcc, w^2; (hchc and hcch): $2wv$; (chhc, chch, cchh), $3v^2$; hccc, w; (chcc, cchc, ccch), $3v$; cccc, 1.

(38) Maison, W.; Kennedy, R. J.; Miller, J. S.; Kemp, D. S. *Tetrahedron Lett.* **2001**, *42*, 4975−4977.

The above L−R state sums were transformed into state sums for the H-bonding cooperativity model as follows. Each noncooperative L−R state sum is a polynomial in v, t, and m, in which v is the weighting used for the helical residues in nonhelical conformations chc and chhc, t is the helix initiation parameter, and w is the alanine helical propensity. For the state sum SS_{cs}, each term within the polynomial containing the coefficient of form t^2w^k corresponds to a conformation that contain a single continuous helical sequence of length k. For the cooperative state sum SS_{te}, all w^k terms that lack a t^j coefficient (j = 2, 4, or 6) correspond to helices that extend to the peptide N-terminus and are initiated by Ac-Hel. Applied to a polynomial y, the Mathematica 3.0 function Coefficient [y,t,2] yields the coefficient for the term in y that contains t^2. Accordingly, (4) converts the state sum SS_{cs} into its cooperative form, and (5) effects the corresponding conversion for SS_{te}. The effect of these transformations is to convert every w^k term in the original state sum into a coop[k] w^k term. (Note that t^4 and t^6 terms are dropped in these calculations, since for plausible values of w, with t = v = 0.048, and with $n \leq 14$, these terms make insignificant contributions to the state sum.) The function coop[k] was assigned the value 1 for $1 \leq k \leq 4$, and $(w')^k$ for $7 \leq k$; coop[5] = $(w')^{(5*1)/3}$; coop[6] = $(w')^{(6*2)/3}$.

$$SS_{cs}\ Cp = \text{Coefficient}[SS_{cs},t,0] + t^2\ \text{sum}(\text{coop}[k]w^k\ \text{Coefficient}[\text{Coefficient}[SS_{cs},t,2],w,k],\{k,3,n\}) \qquad (4)$$

$$SS_{te}\ Cp = \text{Coefficient}[SS_{te},w,0] + \text{sum}(\text{coop}[k]w^k\ \text{Coefficient}[\text{Coefficient}[SS_{te},t,0],w,k],\{k,1,n\}) + t^2\ \text{Sum}(\text{coop}[k]w^k\ \text{Coefficient}[\text{Coefficient}[SS_{te},t,2],w,k],\{k,3,n\}) \qquad (5)$$

Since t and w are used in iteration and symbolic functional transformations, they are assigned numerical values only when these transformations are complete. The parameter v can be assigned its literature value v = 0.048 at the calculation of the state sum. At the end of symbolic transformations, t is assigned the literature value of v = u = 0.048, and w is assigned the value 1.0.

$$\text{ellcs} = (u^2/(n*\text{SScsCp}))\ \text{Sum}(k*\text{theta}[k]w^k\ \text{Coefficient}[\text{Coefficient}[\text{SScsCp},u,2],w,k],\{k,3,n\}) \qquad (6)$$

$$\text{ellte} = (u^2/(n*\text{SSteCp}))\ \text{Sum}(k*\text{theta}[k]w^k\ \text{Coefficient}[\text{Coefficient}[\text{SSteCp},u,2],w,k],\{k,3,n\}) + (1/(n*\text{SScsCp}))\ \text{Sum}(k*\text{theta}[k]w^k\ \text{Coefficient}[\text{Coefficient}[\text{SSteCp},u,0],w,k],\{k,1,n\}) \qquad (7)$$

$$ell_{calc} = \chi_{te}\ \text{ellte} + (1 - \chi_{te})\text{ellcs} \qquad (8)$$

Residue ellipticities for the te and cs conformational substates are calculated from (6) and (7); the mole fraction of the te substate is calculated from (3) as t/c = $A + B(SS_{te}$ coop/SS_{cs} coop), and the mole fraction of te state χ_{te} = (t/c − A)/(1 + t/c); the calculated overall ellipticity is then given by (8), since $1 = \chi_{cs} + \chi_{ts} + \chi_{te}$. In calculating the overall length of the polyalanine region, we regard the ^{t}L residue as a helix stop signal[3] and count the Ac-Hel length as zero. As noted by Qian and Schellman[7c] and Manning and Woody,[8] the nπ* transition of the tertiary proline amides is expected to fall around 230 nm and contribute weakly to the 222 nm CD transition. FH values were calculated analogously by setting the theta[i] terms in (6) and (7) equal to 1.0 and redefining ellcs, ellte, and ellcalc respectively as FHcs, FHte, and FHcalc.

The least-squares minimization of $((t/c)_{exp} - (t/c)_{exp})^2$ was carried out using 2 and 25 °C equations (1), (2), and (3), treating B and w' as variable parameters for each temperature. Gratifyingly, the values of B at the two temperatures are identical (0.177, 0.176) and consistent with previously assigned literature values.

In units of deg-cm^2 dmol^{-1}, theta[k] values used in (5) and (6) are $-61\,000(1 - 2.5/k)$ for $k > 9$, and $-53\,000(1 - 2.5/k)$ for $k \leq 9$.

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Appendix

As noted in the main body of this report, a study of the series Ac-Hel-Ala$_n$ has yielded pairs of data for t/c and $[\theta]_{222}$ at 2, 25, and 60 °C and for $3 \leq n \leq 14$. An excellent linear correlation is observed in Figure 6 between t/c and the function $[\theta]_{222}/([\theta_\infty]_{222} - [\theta]_{222})$ for all data if $[\theta_\infty]_{222}$ is set equal to $-61\,000$. This value for $[\theta_\infty]_{222}$ is currently controversial.[31] In the text we asserted that any likely error in the magnitude of this currently controversial parameter will not degrade the linearity of the correlation significantly. In this Appendix, an analysis of modeling data is presented that justifies this assertion as well as other assumptions implicit in the analysis.

Our approach is to construct a set of values for t/c, FH (fractional helicity), and $[\theta]_{222}$ that are calculated for a plausible range of w, n, and $[\theta_\infty]_{222}$ from the L−R algorithms described in the Experimental Section. Using this simulated data set, we show that a robust linear correlation between t/c and $[\theta]_{222}/([\theta_{test}]_{222} - [\theta]_{222})$ is expected, even when $[\theta_{test}]_{222}$ differs substantially from the value of $[\theta_\infty]_{222}$ used to calculate $[\theta]_{222}$ data points.

Implicit in any $[\theta]_{222}$ correlation is an expected linear relationship between t/c and FH/(1 − FH). This correlation is the proper starting point in this analysis. It can be carried out without knowledge of ellipticity parameters, and it provides insight into important properties of the correlation that can be expected between t/c and $[\theta]_{222}/([\theta_{test}]_{222} - [\theta]_{222})$. The first issues to be resolved are the intrinsic relationships between t/c, FH, and FH/(1 − FH).

What does t/c measure? As noted previously,[4,5] Ac-Hel has a degree of freedom corresponding to s-trans (t) and s-cis (c) conformers of the acetamido function, which equilibrate slowly on the NMR time scale at ambient temperatures. It has a second, ring-derived degree of freedom resulting from rapid equilibration between helical e and nonhelical s states. NMR evidence allows detection of three composite conformational states, cs (nonhelical), ts (nonhelical), and te (helical), and t/c is measured as the ratio of the integrated intensity of the rapidly equilibrating, averaged (ts)−(te) resonance to that of the slowly equilibrating (cs) resonance.[4] The t/c parameter can be expressed as (A1), in which the additive constant A defines the intrinsic t/c bias of the template and the constant B is its tendency to initiate helices in a linked peptide. These empirically assigned parameters are

$$\text{t/c} = ([\text{ts}] + [\text{te}])/[\text{cs}] = [\text{ts}]/[\text{cs}] + [\text{te}]/[\text{cs}] = A + B(\text{te state sum})/(\text{cs state sum}) \quad \text{(A1)}$$

characteristic of the Ac-Hel function and its interactions with the first few residues of the linked peptide. The dependence of t/c on the helical disposition of the linked peptide is thus proportional to a ratio of te and cs state sums. The te state sums are dominated by Ac-Hel-initiated helices, but the cs state sums, which primarily contain terms that correspond to randomly initiated helices,[5] are dominated by weights for random coil conformations. The resulting t/c ratio is to a reasonable approximation proportional to a sum of helical te weights, one for each helical conformer of length $\leq n$ that is initiated at the peptide−Ac-Hel junction. The magnitude of t/c shows a significant increase either for elongation of the peptide or for an increase in its intrinsic helical propensity w. The potential range of t/c is 0 to ∞.

What do FH and $[\theta]_{222}$ measure? As seen in (A2), $[\theta]_{222}$ is usually taken to be proportional to FH, which is defined as the ratio of helical peptide α-carbons to the total number of α-carbons that are potentially helical. The range of FH is thus 0 to 1.0, and for highly helical peptides, it is relatively insensitive to increases in length or helical propensity. As seen from (A3), $[\theta]_{222}$, for a particular peptide length n, converges to a limiting value $[\theta_n]_{222}$ as FH approaches 1.0. Given that t/c reflects a different range and different sensitivities to length and w, t/c is not expected to correlate linearly with either FH or $[\theta]_{222}$. These parameters or t/c itself must be transformed to render the ranges compatible.

$$[\theta]_{222} \approx [\theta_n]_{222}\text{FH} \quad \text{(A2)}$$

$$[\theta_n]_{222} = [\theta_\infty]_{222}(1 - x/n) \quad \text{(A3)}$$

where

n is the length of the potentially helical region

$[\theta_n]_{222}$ is the residue ellipticity of an idealized 100% helical peptide of length n

$[\theta_\infty]_{222}$ defines the limit of function (A3) for very large n

x corrects for end effects

For two reasons we have chosen to correlate t/c with a transformed FH or $[\theta]_{222}$. First, since t/c is the quantity that is being tested for its relationship to more conventional helicity standards, a correlation seems to us more convincing if the experimental values for t/c are tested without mathematical manipulation. Second, as noted above, t/c is roughly proportional to the helical state sum, which is the most fundamental quantity to emerge from mathematical modeling. As such it gives a better intuitive picture of helix stabilization, particularly for highly helical peptides.

The function FH/(1 − FH) defines the ratio of peptide helical to nonhelical α-carbons in the peptide. A closely related ratio is $\chi_{Hel}/(1 - \chi_{Hel})$, which is the mole fraction of helical to nonhelical conformations in the peptide. This ratio correlates with the state sum ratio of (1) and thus is approximately proportional to t/c. It is simple to show that FH/(1 − FH) correlates with $\chi_{Hel}/(1 - \chi_{Hel})$ but is invariably smaller.[39]

(39) The anticipated correlation and inequality can be quantitatively modeled from the equality $\text{FH} = \sum(k/n)\chi_{Hel-k}$, where χ_{Hel-k} is the mole fraction of helical conformations of length k, $3 \leq k \leq n$. Then $\chi_{Hel} = \sum\chi_{Hel-k}$, the mole fraction of all helical conformations, and $1 = \chi_{Hel} + \chi_{NonHel}$, and $\chi_{Hel}/(1 - \chi_{Hel}) = \chi_{Hel}/\chi_{NonHel}$. $\text{FH}/(1 - \text{FH}) = \sum(k/n)\chi_{Hel-k}/(\chi_{Hel} + \chi_{NonHel} - \sum(k/n)\chi_{Hel-k}) = \sum(k/n)\chi_{Hel-k}/(\sum\chi_{Hel-k} + \chi_{NonHel} - \sum(k/n)\chi_{Hel-k}) = \sum(k/n)\chi_{Hel-k}/(\chi_{NonHel} + \sum((n - k)/n)\chi_{Hel-k})$. Since $n \geq k$, $\sum(k/n)\chi_{Hel-k} < \sum\chi_{Hel-k}$, and the numerator of FH/(1 − FH) is less than the numerator of $\chi_{Hel}/(1 - \chi_{Hel})$. Also, $\sum((n - k)/n)\chi_{Hel-k} > 0$, so the denominator of FH/(1 − FH) is greater than that of $\chi_{Hel}/(1 - \chi_{Hel})$. Accordingly, $\text{FH}/(1 - \text{FH}) < \chi_{Hel}/(1 - \chi_{Hel})$, but the two ratios contain many common terms and thus correlate.

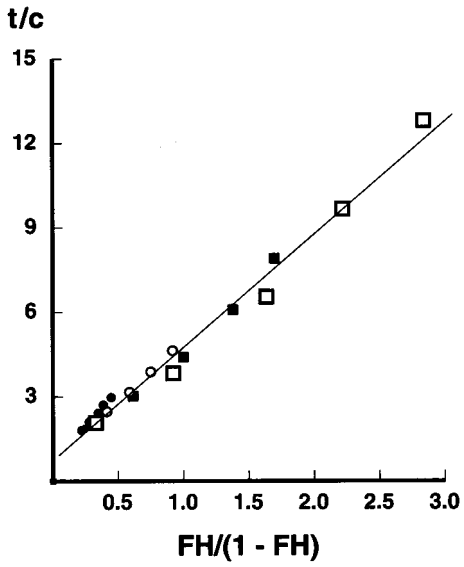

Figure 8. Test of the linearity of a plot of t/c and FH data calculated for a L−R model with peptide lengths 6, 8, 10, 12, and 14, and for w' values of 1.05 (●), 1.15 (○), 1.25 (■), and 1.35 (□). As noted in the text, all modeled data points fit a linear correlation with only minor systematic deviations. Although the cooperative L−R model (using w' values) developed in the text was used, equivalent results are obtained with a conventional L−R model.

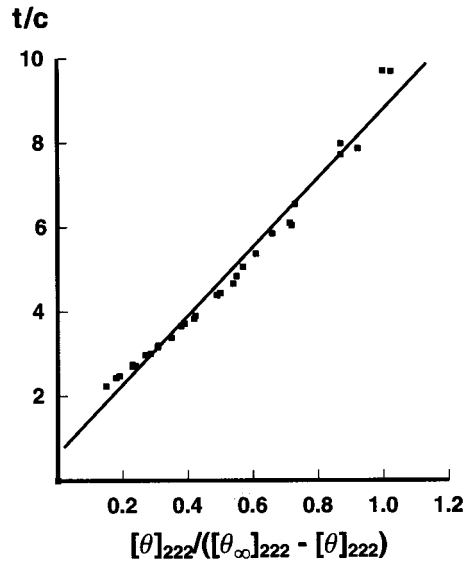

Figure 9. Test of relationship (4) and the linearity of t/c vs $[\theta]_{222}/([\theta_\infty]_{222} - [\theta]_{222})$ plots. The following L−R series gave t/c ≤ 10: $n = 10, 12, 14$ for $w' = 1.05, 1.10, 1.15, 1.20$; $n = 8, 10, 12, 14$ for $w' = 1.25$; $n = 8, 10, 12$ for $w' = 1.30, 1.35$; $n = 6, 8, 10$ for $w' = 1.45$. Each $[\theta]_{222}$ series was calculated in turn using one of the four values of $[\theta_\infty]_{222}$ reported in the text; this value was then used to calculate $[\theta]_{222}/([\theta_\infty]_{222} - [\theta]_{222})$ data points for that series.

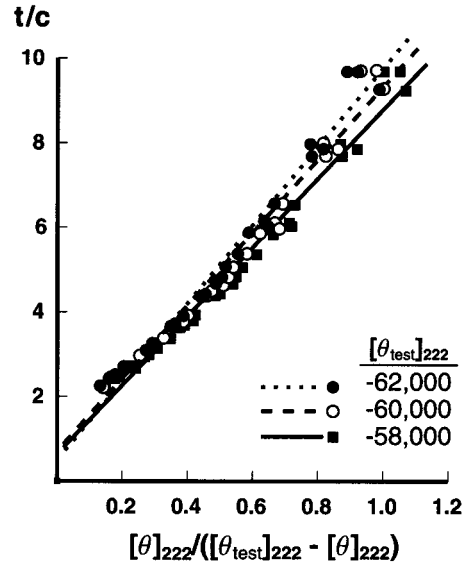

Figure 10. Plot of t/c and $[\theta]_{222}$ data calculated as described in Figure 9 using $-62\,000 = [\theta_\infty]_{222}$. The correlation of greatest slope corresponds to a calculation using −62 000 for $[\theta_{test}]_{222}$ in the calculation of $[\theta]_{222}$ and to transform the data to $[\theta]_{222}/([\theta_{test}]_{222} - [\theta]_{222})$. For the line of least slope, the previous $[\theta]_{222}$ data were transformed setting $[\theta_{test}]_{222} = -58\,000$ (6.5% lower), and for the center line, $[\theta_{test}]_{222} = -60\,000$ was used in the transformation. For this small range of error, the quality of the linear correlation is unchanged, and the model data points lie within likely measurement error.

Modeling shows that FH/(1 − FH) and t/c are not expected to exhibit a good linear correlation over an extended t/c range in which FH ⟶ 1.0. The critical question is, do they correlate satisfactorily over the experimental range for which t/c can be characterized with adequate precision? Since t/c is a ratio of integrated NMR resonances, this range is roughly 1 to 10.

Figure 8 shows a typical correlation of t/c and FH/(1 − FH) that results from L−R-derived data. In generating the data set for Figure 8, we varied peptide length n from 6 to 14 and, for each length in this range, varied the helical propensity w from 1.05 to 1.35. This change in w approximates the experimental change in temperature from 2 to 60 °C. The remarkable feature of the graph is that all points calculated for very different values of n and w lie close to the line of best linear fit, with an overall correlation coefficient (CC) of 0.989. FH/(1 − FH) is unique in correlating well with t/c. For example, in the same data set, correlating t/c with length alone yields CC = 0.70, and CC for the correlation of t/c with w alone is 0.48.

The limits of the effective correlation were also explored. When the modeling database was expanded to include points for $w' > 1.40$, $n > 10$, somewhat greater scatter in the plot was observed, but the slope of the line of best fit remained near the value of 4.0 that is seen in Figure 8. The correlation between t/c and FH/(1 − FH) is not perfect, as evidenced by the finite intercept and the slight curvature that is seen within a series of constant w'. Both effects are anticipated. The positive intercept is attributable primarily to the presence of the nonhelical ts state, which makes a constant contribution to t/c. The curvature is primarily attributable to the length-dependent change of the effect of the k/n weighting.[39] The slope of the graph is significantly higher than 1.0, primarily because of the k/n weighting and the variation in its contribution to high and low FH data.

The ellipticity analogue of FH/(1 − FH) is the equation $[\theta]_{222}/([\theta_\infty]_{222} - [\theta]_{222})$, which depends not only on the experimental ellipticity $[\theta]_{222}$ but also on the limiting molar residue ellipticity $[\theta_\infty]_{222}$. Remarkably, (A4) shows that substitution of (A2) and (A3) into the expression $[\theta]_{222}/([\theta_\infty]_{222} - [\theta]_{222})$ allows elimination of $[\theta_\infty]_{222}$ and transformation into an analogue of FH/(1 − FH) in which 1 is replaced by $n/(n - x) > 1$, where x is a length correction that is often assigned a value 2.5.[29] This result implies that data derived from more than one peptide series, each characterized by its own value of $[\theta_\infty]_{222}$ will yield a linear

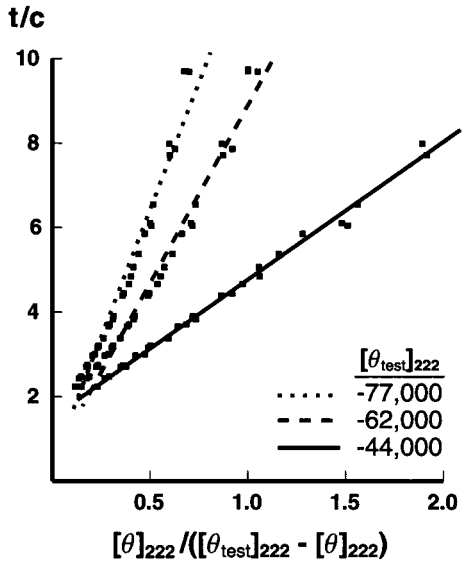

Figure 11. Same analysis as in Figure 10, but with a larger change in $[\theta_{test}]_{222}$: correlation of greatest slope, $[\theta_{test}]_{222} = -77\,000$; correlation of least slope, $[\theta_{test}]_{222} = -44\,000$; center line, $[\theta_\infty]_{222} = -62\,000$. The quality of linear correlation is changed only slightly (CC = 0.97, 0.98. 0.99), but the slope is markedly dependent on the choice of $[\theta_{test}]_{222}$.

graph, provided the appropriate value of $[\theta_\infty]_{222}$ is applied to data from each series when calculating $[\theta]_{222}/([\theta_\infty]_{222} - [\theta]_{222})$.

$$[\theta]_{222}/([\theta_\infty]_{222} - [\theta]_{222}) \approx \mathrm{FH}/(n/(n - x) - \mathrm{FH}) \quad \text{(A4)}$$

As seen in Figure 9, modeling confirms this conclusion. For $n = 3-14$, and $w' = 1.05-1.45$, using $-[\theta_\infty]_{222} = 30$, 44, 62, and 77,[40] respective zero intercepts were 0.79, 0.73, 0.75, and 0.75, with respective slopes of 8.5, 8.6, 8.6, and 8.55. These differences would be experimentally indistinguishable.

As noted in the text, it appears likely that the value of $-[\theta_\infty]_{222}$ for alanine-rich peptides is significantly larger than values characteristic of typical helices found in folded proteins, leucine zippers, and coiled coils. What happens to the correlation of Figure 9 if the value of $[\theta_{test}]_{222}$ used in the calculation of $[\theta]_{222}/([\theta_{test}]_{222} - [\theta]_{222})$ is too large or too small? The comparisons of Figures 10 and 11 show that very little change occurs in the quality of linear correlation, but substantial changes occur in slope. The quality of the linear correlation remains unchanged because in these examples the value of $-[\theta_{test}]_{222}$ is always at least 20% larger than $-[\theta]_{222}$. False, very large values of $[\theta]_{222}/([\theta_{test}]_{222} - [\theta]_{222})$ are expected if the magnitude of $[\theta_{test}]_{222}$ is grossly small and $[\theta]_{222}$ converges to it because FH approaches 1.0.

This analysis proves that a correlation of t/c data with peptide length or with $[\theta]_{222}$ itself (compare data of Figures 5 and 7) is found to be decidedly nonlinear, as predicted from modeling experiments. By contrast, strikingly linear plots are observed if t/c is plotted as a function of FH/(1 − FH) or $[\theta]_{222}/([\theta_{test}]_{222} - [\theta]_{222})$. This linearity holds for a range of lengths and helical propensities, provided that $[\theta_{test}]_{222}$ approximates $[\theta_\infty]_{222}$ within wide limits.

We conclude that the linearity of Figure 6, which was observed for experimental data, implies that both t/c and $[\theta]_{222}$ model intrinsic peptide helicity. When the value of $[\theta_\infty]_{222}$ for alanine-rich peptides is experimentally determined, t/c can be calculated from $[\theta]_{222}$ and vice versa. Figure 6 thus validates our use of t/c as a quantitative helicity measure.

Supporting Information Available: Compound characterization including analytical ultracentrifugation, NHND exchange, and circular dichroism data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA016285C

(40) In units of 10^{-3} deg cm^2 dmol^{-1}.