Nonpolar contributions to conformational specificity in assemblies of designed short helical peptides

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(RECEIVED December 15, 1999; FINAL REVISION March 20, 2000; ACCEPTED March 23, 2000)

Abstract

A series of designed short helical peptides was used to study the effect of nonpolar interactions on conformational specificity. The consensus sequence was designed to obtain short helices (17 residues) and to minimize the presence of interhelical polar interactions. Furthermore, the sequence contained a heptad repeat (abcdefg), where positions a and d were occupied by hydrophobic residues Leu, Ile, or Val, and positions e and g were occupied by Ala. The peptides were named according to the identities of the residues in the adeg positions, respectively. The peptides llaa, liaa, ilaa, iiaa, ivaa, viaa, lvaa, vlaa, and vvaa were synthesized, and their characterization revealed marked differences in specificity. An experimental methodology was developed to study the nine peptides and their pairwise mixtures. These peptides and their mixtures formed a vast array of structural states, which may be classified as follows: helical tetramers and pentamers, soluble and insoluble helical aggregates, insoluble unstructured aggregates, and soluble unstructured monomers. The peptide liaa formed stable helical pentamers, and iiaa and vlaa formed stable helical tetramers. Disulfide cross-linking experiments indicated the presence of an antiparallel helix alignment in the helical pentamers and tetramers. Rates of amide proton exchange of the tetrameric form of vlaa were 10-fold slower than the calculated exchange rate for unfolded vlaa. In other work, the control of specificity has been attributed to polar interactions, especially buried polar interactions; this work demonstrated that subtle changes in the configuration of nonpolar interactions resulted in a large variation in the extent of conformational specificity of assemblies of designed short helical peptides. Thus, nonpolar interactions can have a significant effect on the conformational specificity of oligomeric short helices.

Keywords: conformational specificity; de novo design; helical bundles; nonpolar interactions; peptide assemblies

The hydrophobic interaction is considered to be a dominant force in stabilizing proteins (Dill, 1990). However, the contribution of hydrophobic interactions to conformational specificity or structural uniqueness is under debate. Early attempts at protein design were based on the belief that the hydrophobic interaction could encode both stability and specificity in protein structure. The first design targets were helical bundles; and the design principle used a simple sequence pattern to produce a bundle of amphipathic helices, with an exclusively hydrophobic core and minimal exposure of nonpolar amino acids to solvent (Ho & DeGrado, 1987).

Synthesis and characterization of the early designed peptides revealed that they were very stable to thermal and chemical denaturation; however, the side chains were not close-packed, and thus lacked the conformational specificity typical of a native protein (Handel et al., 1993). After a series of design modifications, where an increasing number of polar interactions were introduced into the core, a four-helix bundle with native-like properties was produced (Hill & DeGrado, 1998). Thus, while hydrophobic interactions imparted stability to the designed proteins, they were insufficient for the formation of a specific structure. These protein design studies have led to the idea that buried polar interactions in the hydrophobic core contribute more to conformational specificity than hydrophobic interactions.

The ability of buried polar interactions to cause an increase in conformational specificity has also been highlighted in studies of coiled coils. In the dimeric state of the naturally occurring coiled coil motif of GCN4, there is an Asn residue that lies in the nonpolar dimer interface. This Asn forms an interhelical, side-chain–side-chain hydrogen bond with the other corresponding Asn side chain in the dimer (O’Shea et al., 1991). Mutation of the Asn to a nonpolar amino acid resulted in a loss of conformational specificity.

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Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonic acid; CD, circular dichroism; DMF, *N,N*-dimethylformamide; ES-MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HP, H: hydrophobic, P: polar; HPLC, high-performance liquid chromatography; PAL-PEG-PS, 5-(aminomethyl-3',5'-dimethoxyphenoxy) valeric acid polyethylene glycol polystyrene-graft copolymer; UV, ultraviolet.

ity (Harbury et al., 1993; Lumb & Kim, 1995). Similar results were obtained in studies of peptide Velcro, a designed coiled coil peptide based on the sequence of GCN4 (O'Shea et al., 1993). Substitution of Leu for Asn at position 14 in the hydrophobic core of peptide Velcro led to an increase in stability, but a loss of specificity. From these results, it was concluded that nondirectional hydrophobic interactions contribute to protein stability, but do not impart conformational specificity (Lumb & Kim, 1995).

An opposing view arises from theoretical approaches, employing HP models, which predict that structural uniqueness can be encoded by hydrophobic interactions (Dill et al., 1995). Experimental support for this idea comes from the work of Roy et al. (1997). The Roy group designed a protein with native-like properties, whose sequence was based on a "binary pattern" of polar and nonpolar amino acids with a predominantly hydrophobic core (Roy et al., 1997). The Regan group redesigned the hydrophobic core of Rop to contain Leu and Ala residues, exclusively (Munson et al., 1996). The redesigned Rop had native-like properties. These studies demonstrate that it is possible to construct proteins having exclusively hydrophobic cores and also possess conformational specificity.

Many of the studies mentioned above made use of coiled coils whose helical segments were relatively long (~30 residues). However, helices in globular proteins are usually less than 20 residues long, with an average length of 11 residues. Current data suggest that the behavior of coiled coil assemblies may differ from short helical bundles. For example, while helices of coiled coil proteins are usually parallel (exceptions: Monera et al., 1993; Oakley & Kim, 1997), to date, the crystal structures of all bundles of short helices indicate an antiparallel alignment (Schafmeister et al., 1993; Taylor et al., 1996; Prive et al., 1999). Another difference is that short helical assemblies are not dimeric, but usually contain three or more helices. Therefore, the mechanism through which conformational specificity is conferred may be different for shorter helices. Consequently, we have chosen to investigate how nonpolar interactions contribute to specificity in assemblies of short helices (17 residues) and compare the results with coiled coil studies.

A further distinction of all the above-mentioned studies is that they use model proteins, which contain numerous polar interactions involving hydrophilic side chains. The presence of these

interactions may mask the contributions of nonpolar interactions to conformational specificity. To avoid this complication, we have designed a simplified system for examining the specificity and stability of helix associations. This system contains minimal interhelical polar interactions so that helix association is driven by nonpolar interactions involving relatively few amino acid residues. Our major finding is that subtle changes in the number and type of nonpolar interactions resulted in a distribution of states ranging from unstructured aggregates to specific oligomeric states. Although we did not find structurally unique species within this distribution, we did observe states with vast differences in their conformational specificity.

Results

Peptide design

Table 1 shows the sequences of the peptides designed for this study. The consensus sequence, given by Ac-DdeQgaKQdeEgaQKdeGGY-amide, was designed to obtain helices 17 residues long containing a heptad repeat, i.e., seven residues (labeled a, b, c, d, e, f, g) that recur in the sequence. A heptad repeat is commonly found in the sequence of many naturally occurring coiled-coil and α -helix bundle-containing proteins (Cohen & Parry, 1990). Usually, heptad positions a and d are occupied by hydrophobic amino acids, whereas positions e and g are occupied by charged amino acids. In this work, the name of each peptide was derived from the identities of the amino acids in the a, d, e, g positions, respectively. For example, the peptide vlaa has Val in position a, Leu in position d, and Ala in positions e and g. Because of this nomenclature, the peptide series as a whole is called the adeg series. Upon folding, the presence of the heptad repeat in the consensus sequence will result in the formation of amphipathic helices, thereby favoring oligomerization. Figure 1 shows a helical wheel diagram of the peptide llaa and illustrates the amphipathic nature of the adeg peptides.

To study the contribution of nonpolar interactions to conformational specificity of short helical bundles, the peptides were designed to minimize the impact of polar interactions on specificity. Interhelical polar interactions occurring between amino acids occupying e and g heptad positions are known to affect specificity

Table 1. Sequences of the adeg^a peptides

Name	Sequence and heptad positions																			
	c	d	e	f	g	a	b	c	d	e	f	g	a	b	c	d	e			
llaa	D	L	A	Q	A	L	K	Q	L	A	E	A	L	Q	K	L	A	G	G	Y
liaa	D	I	A	Q	A	L	K	Q	I	A	E	A	L	Q	K	I	A	G	G	Y
ilaa	D	L	A	Q	A	I	K	Q	L	A	E	A	I	Q	K	L	A	G	G	Y
iaaa	D	I	A	Q	A	I	K	Q	I	A	E	A	I	Q	K	I	A	G	G	Y
ivaa	D	V	A	Q	A	I	K	Q	V	A	E	A	I	Q	K	V	A	G	G	Y
vial	D	I	A	Q	A	V	K	Q	I	A	E	A	V	Q	K	I	A	G	G	Y
lvaa	D	V	A	Q	A	L	K	Q	V	A	E	A	L	Q	K	V	A	G	G	Y
vlaa	D	L	A	Q	A	V	K	Q	L	A	E	A	V	Q	K	L	A	G	G	Y
vvaa	D	V	A	Q	A	V	K	Q	V	A	E	A	V	Q	K	V	A	G	G	Y

^aThe name of each peptide is derived from the identity of the residues in the a, d, e, g, heptad positions, respectively. The name adeg is used to refer to the peptides collectively.

Fig. 1. Helical wheel diagram of llaa. The view is from the N-terminus, down the helical axis. The heptad positions are contained in circles labeled a–g, and they span the first two turns of the helix. The sequence of llaa begins at heptad position c. The other adeg peptides differ only by the identity of the residues in the a and d heptad positions (see Table 1 for sequences of the adeg peptides).

and, to a lesser extent, stability (O'Shea et al., 1993; Kohn et al., 1995; Nautiyal et al., 1995; Zeng et al., 1997). Thus, Ala was chosen to occupy the e and g heptad positions to prevent inter-helical polar interactions at these positions. The a and d positions, comprising the hydrophobic core, were occupied by the nonpolar amino acids Leu, Ile, or Val in all possible combinations (nine peptides in total). In addition, Asp was chosen for the N-terminus to stabilize helix formation, because (1) it is known to have a preference for the N-cap position in naturally occurring helices (Richardson & Richardson, 1988) and (2) it provides a favorable electrostatic interaction with the helix dipole (Shoemaker et al., 1987). The internal heptad positions b, c, and f are occupied by Gln because it is a polar uncharged amino acid with a high helix propensity (Chakrabarty & Baldwin, 1995), features that will aid in peptide solubility, formation of an amphipathic helix, and helix stability. Moreover, an (*i*, *i* + 4) Glu-Lys salt bridge was designed into the sequence because it has been shown to stabilize helix formation in short peptides (Marqusee & Baldwin, 1987).

Last, Tyr appears in the consensus sequence to facilitate concentration determination by UV absorbance. Two Gly residues link the Tyr to the rest of the sequence to prevent a contribution from Tyr to the circular dichroism (CD) signal of the peptides (Chakrabarty et al., 1993). In addition, the two Gly residues, by virtue of their helix breaking activity (Chakrabarty & Baldwin, 1995), position the Tyr outside of the helical segment. Moreover, in previous work by Lovejoy et al. (1993), a Trp residue was incorporated into the sequence of a designed coiled coil named coil-Ser, at the N-terminus, to facilitate UV spectroscopic studies. However, the crystal structure of coil-Ser indicated that it formed antiparallel trimers because the bulky side chain of Trp was best accommodated in this structure (Lovejoy et al., 1993). Thus, the presence of Trp within the helix actively affected the oligomerization of coil-Ser. Because the Gly linkers in the consensus sequence of the adeg series leave Tyr out of the helix, it is unlikely that Tyr interfered with oligomerization in this system.

Determining presence of specific oligomers in peptide mixtures

We examined two types of oligomers in the adeg series: (1) homo-oligomers of each of the adeg peptides (nine in total), and (2) hetero-oligomers that would arise from all possible mixtures of the adeg peptides (36 in total). To improve the efficiency of characterization of the 45 samples, we devised five experiments to rapidly screen the samples. Because we want to study specific oligomers, these tests allowed us to eliminate from further analysis those peptides and mixtures that were predominantly unfolded and those that formed nonspecific aggregates. Furthermore, the tests provided insight into the secondary structure and conformational specificity of the peptides. All the screening tests were performed using 50 μ M total peptide concentration in buffer (10 mM Na_2HPO_4 , 120 mM NaCl, pH 7), and the results of each test are given in matrix form.

The first test examined the solubilities of the adeg peptides and mixtures. The samples were centrifuged and then the UV absorbances of the supernatants were measured. This experiment showed which peptides and mixtures formed insoluble nonspecific aggregates and eliminated them from further characterization. Figure 2A is a summary of the results of the solubility test. This experiment indicated that the peptides llaa, ivaa, and viaa were the only peptides that formed insoluble aggregates. The peptides liaa, ilaa, iiaa, lvaa, vlaa, vvaa, and their mixtures were 100% soluble.

After UV absorbance measurements were performed, the same samples were studied using light scattering. This method allowed us to identify large soluble aggregates present in the peptide solutions. Because the samples had peptide concentrations $\leq 50 \mu\text{M}$, only large aggregates would scatter enough light to be detected. The matrix in Figure 2B summarizes the results of the light-scattering experiment. The peptides llaa and ilaa had high scattering intensities due to the presence of large aggregates in solution. Quantitative analysis of dynamic light scattering data from the ilaa stock sample (1.5 mM) indicated that $>90\%$ of the molecular species detected had a hydrodynamic radius of ~ 20 nm. The results shown in Figure 2A indicate that llaa also formed insoluble aggregates, whereas ilaa was 100% soluble. Therefore, ilaa formed large soluble aggregates and llaa formed both soluble and insoluble aggregates. The remaining adeg peptides showed insignificant scattering intensities.

After establishing the solubility and relative sizes of the adeg homo- and hetero-oligomers, their structure was investigated using CD spectroscopy. CD spectroscopy was used to distinguish between folded and unfolded structures, and to determine the nature of the folded states of the adeg peptides. Figure 3A shows that the peptides llaa, liaa, ilaa, iiaa, and vlaa had spectra characteristic of α -helices with minima at the wavelengths 222 and 208 nm. Figure 3B shows that ivaa, viaa, lvaa, and vvaa had spectra characteristic of the random coil or unfolded state. Because the peptides seemed to be either helical or random coil, a wavelength of 222 nm was chosen to compare the secondary structures of all the peptides and mixtures.

Figure 2C shows the matrix that summarizes the results of the CD screening experiment. The results indicate that liaa had the highest helix content, followed by ilaa and llaa; iiaa and vlaa had an intermediate helix content, and ivaa, viaa, lvaa, and vvaa had the lowest helix content. Furthermore, these data are consistent with the results of the light-scattering experiment. The peptide llaa formed insoluble nonspecific helical aggregates; ilaa formed sol-

Fig. 2. **A:** Solubility of adeg peptide mixtures. The 45 samples were centrifuged, and the UV absorbance of the supernatants was measured at 275 nm (A_{275}) to determine peptide solubility. All samples consisted of 50 μ M total peptide in buffer containing 10 mM Na_2HPO_4 , 120 mM NaCl, pH 7 at 25 °C. A soluble peptide solution at a concentration of 50 μ M should have $A_{275} = 0.0695$. The peptides were considered to be 0% soluble when $A_{275} < 0.025$ (square containing an "X"), 50% soluble when $A_{275} = 0.025$ –0.045 (square containing a "\"), and 100% soluble when $A_{275} = 0.060$ –0.075 (empty square). **B:** Light-scattering properties of adeg peptide mixtures. The right angle-scattering intensity was measured and normalized to obtain the relative scattering intensity using $(x_i - x_{\min})/(x_{\max} - x_{\min})$, where x_i is the scattering intensity of peptide mixture i , $x_{\min} = 4 \times 10^3$ counts/s, and $x_{\max} = 2,490 \times 10^3$ counts/s. The relative scattering intensity values were then applied to a gray scale for shading. **C:** Helix content of adeg peptide mixtures. All measurements were performed in buffer containing 10 mM Na_2HPO_4 , 120 mM NaCl, pH 7 at 25 °C. $[\theta]_{222}$ was used as an indication of helix content. The shading indicates the relative helix content of the adeg peptides and mixtures, and was calculated as in Figure 2B. In this case, $x_{\min} = 2$ k and $x_{\max} = 26$ k (where k = 10^3 deg cm²/dmol). **D:** ANS binding of adeg peptide mixtures. The relative fluorescence (F_R) of each peptide mixture was calculated from the fluorescence of a peptide sample containing ANS ($F_{\text{pep}+\text{ANS}}$), and the fluorescence of ANS alone (F_{ANS}), using $F_R = F_{\text{pep}+\text{ANS}}/F_{\text{ANS}}$. These values were then normalized to fall between zero and one as in **B** and applied to a grey scale.

Fig. 3. CD spectra of the adeg peptides. All measurements were performed in buffer containing 10 mM Na_2HPO_4 , 120 mM NaCl, pH 7 at 25 °C. The helical peptides are (A) $\blacklozenge$, vlaa 75 μM ; $\circ$, llaa 75 μM ; ∇ , liaa 76 μM ; $\blacksquare$, ilaa 75 μM ; $\square$, ilaa 75 μM . The unfolded peptides are (B) $\blacksquare$, viaa 75 μM ; $\square$, vvaa 69 μM ; $\blacklozenge$, lvaa 100 μM ; ∇ , lvaa 75 μM .

uble nonspecific helical aggregates; lvaa and vvaa had a low scattering intensity because they were unfolded; liaa was soluble and helical, but may form some large aggregates. However, ilaa and vlaa may form specific helical oligomers because they did not form soluble or insoluble aggregates.

After the CD screen, the same samples were used for an ANS (1-anilinonaphthalene-8-sulfonic acid) dye binding experiment, whose results are summarized in Figure 2D. ANS is a dye whose fluorescence increases when it is sequestered in a hydrophobic environment and has been used to identify exposed hydrophobic surfaces in proteins. Furthermore, molten globule structures also show ANS binding (Kuwajima, 1989). The helical peptide liaa showed a large increase in ANS fluorescence, illustrating that this folded peptide had an exposed hydrophobic region in which ANS was bound. The peptides ilaa and vlaa, respectively, showed an intermediate and small increase in ANS fluorescence, which may be indicative of a more native-like character than that possessed by liaa. The peptides llaa, ilaa, lvaa, and viaa, which form nonspecific aggregates, showed an increase in ANS fluorescence indicating that there are exposed clusters of hydrophobic residues in the aggregates. Last, the unfolded peptides lvaa and vvaa did not show an increase in ANS fluorescence.

Using the screening tests, we eliminated peptides that were unstructured, insoluble, and prone to aggregation, and we identified

peptides that may form specific helical oligomers. The four tests (1) proved to be an efficient means of screening for particular properties from a large number of similar peptides, and (2) provided important information about the structure and specificity of the peptides. Based on the results of the screening tests, we have identified liaa, ilaa, and vlaa as peptides that potentially form specific helical oligomers. In addition, the tests indicated that viaa and lvaa formed insoluble, unstructured aggregates; llaa and ilaa formed insoluble and soluble helical aggregates, respectively; and lvaa and vvaa formed soluble, unstructured monomers.

Information regarding the presence of specific stable hetero-oligomers was obtained using the helix content data (Fig. 2C), ANS fluorescence data (Fig. 2D), and the following method. If hetero-oligomers did not form, then the helix content (or ANS fluorescence) of any given pairwise mixture would be equal to the average of the helix content (or ANS fluorescence) of the two constituent peptides. We measured the percent deviation of the observed helix content (or ANS fluorescence) of peptide mixtures from the calculated average. Percent deviations greater than 10% of both the helix content and the ANS fluorescence were deemed significant. Using these criteria, the only peptide mixture that seemed to form hetero-oligomers was llaa + ilaa. However, the mixture llaa + ilaa contained some insoluble material, and consequently, it was not investigated further. This method would not highlight mixtures containing similar proportions of homo- and hetero-oligomers. However, we were only interested in identifying mixtures solely containing hetero-oligomers, which would be highlighted by this method.

Oligomerization state

The screening tests identified the peptides ilaa, vlaa, and liaa as candidates for specific oligomer formation. Subsequently, the oligomerization states of the peptides were determined using sedimentation equilibrium ultracentrifugation. Sedimentation equilibrium analysis was performed for each peptide by simultaneously fitting all the data taken at different concentrations and different rotor speeds to the sedimentation equilibrium equation for a single ideal species. For the peptides ilaa, vlaa, and liaa (Fig. 4), the analysis indicated a number-average molecular weight of 8,277, 8,603, and 11,275 Da, respectively. The corresponding 95% confidence intervals were 7,755–8,806 Da for ilaa, 7,561–9,666 Da for vlaa, and 10,073–12,469 Da for liaa. These data suggested that both ilaa and vlaa form tetramers, while liaa forms pentamers. The percentage difference between the number-average molecular weight and the calculated oligomeric molecular weight is 3, 2, and 5%, respectively, for ilaa, vlaa, and liaa. We did not observe such a decrease. To confirm our assignment of the oligomeric state of the peptides, the data for each peptide were also fit to various association models. The goodness of the different fits were assessed by comparing the randomness of the residuals, the magnitudes of the variance estimates, and whether the association constants seemed physically plausible. Based on these criteria, the simplest model that best described the data was a stable tetramer for ilaa and vlaa, and a stable pentamer for liaa. However, the presence of a small amount of oligomeric species smaller than a tetramer cannot be ruled out.

Stability of the adeg oligomers

To further characterize the specific adeg peptides, the variation of helix content with concentration of liaa, ilaa, and vlaa was exam-

Fig. 4. Sedimentation equilibrium ultracentrifugation of iiaa, liaa, and vlaa. Data shown is at 100 μ M total peptide concentration in buffer containing 10 mM Na_2HPO_4 , 120 mM NaCl, pH 7 at 20 $^\circ\text{C}$ for the peptides (A) iiaa: 25,000 rpm, 230.5 nm; (B) liaa: 26,500 rpm, 275 nm; and (C) vlaa: 31,000 rpm, 275 nm. The curves represent the best fit of the data to the sedimentation equilibrium equation for a single ideal species.

Fig. 5. Concentration dependence of the helix content of the adeg peptides. $[\theta]_{222}$ was used as an indication of helix content. The $[\theta]_{222}$ of (●) iiaa was obtained in ddH_2O at 25 $^\circ\text{C}$, pH 3.15. The spectra of peptides (+) liaa, (∇) iiaa, (○) vvaa, (◆) vlaa, and (■) liaa were obtained in 10 mM Na_2HPO_4 , 120 mM NaCl, pH 7 at 25 $^\circ\text{C}$.

ined using CD spectroscopy. In addition, the concentration dependence of the helix content of liaa, iiaa, and vvaa was investigated to compare the stabilities of the specific and nonspecific adeg peptides. Figure 5 shows the concentration dependence of the ellipticity of liaa, liaa, iiaa, vlaa, and vvaa at 222 nm, which was used as an indication of helix content (as described earlier). To quantitate the dissociation constants of the specific adeg peptides, the data in Figure 5 for iiaa, vlaa, and iiaa were fit to a version of the Hill equation, modified to model oligomerization (DeGrado & Lear, 1985), using a nonlinear least-squares fit. The goodness of the fits was determined by minimizing on the sum of the squares of the residuals given by $\Sigma(\theta_{\text{obs}} - \theta_{\text{calc}})^2$. Because the sedimentation equilibrium results indicated that iiaa and vlaa form tetramers, a monomer–tetramer model was used to fit the data from Figure 5 for iiaa and vlaa. The curve fitting results for iiaa and vlaa are shown in Figures 6A and 6B, respectively. The concentrations at which iiaa and vlaa are 50% unfolded are 26 and 50 μ M, respectively. Because liaa was found to form pentamers using sedimentation equilibrium analysis, a monomer–pentamer model was used to fit the CD data for liaa from Figure 5. The curve fit results for liaa are shown in Figure 6C. Moreover, the concentration at which liaa was 50% unfolded was 16 μ M. The results obtained from the curve fitting suggest that liaa formed the most stable oligomers followed by iiaa and vlaa.

Assessment of parallel vs. antiparallel alignment of helices in adeg oligomers

The final step in the characterization of the adeg oligomers was to assess whether the helical bundles contained helices in a parallel or antiparallel orientation. The alignment of the helices was determined through a disulfide cross-linking experiment employing two cysteine-containing peptides: one with an N-terminal cysteine and the other with a C-terminal cysteine. To ensure that the cysteine was external to the helix, the cysteine residues were appended onto the sequences of liaa, iiaa, and vlaa using a diglycine linker. The new peptides were called cys-adeg or adeg-cys, according to whether the cysteine residue was located at the N- or C-terminus, respectively. In addition, the adeg-cys peptides contained an extra

Fig. 6. Stability of iiaa, vlaa, and liaa oligomers. The concentration dependence of the helix content of iiaa, vlaa, and liaa were fit to a modified version of the Hill equation. The data for the peptides (A) iiaa and (B) vlaa were fit to the equation for a tetramer model. The dissociation constants for iiaa and vlaa were calculated to be $1.80 \pm 0.48 \times 10^{-14} \text{ M}^3$ and $1.22 \pm 0.43 \times 10^{-13} \text{ M}^3$, respectively. The data for (C) liaa were fit to a pentamer model, and the dissociation constant was calculated to be $6.15 \pm 0.97 \times 10^{-20} \text{ M}^4$.

Table 2. Sequences of peptides used for determining the alignment of adeg helical bundles

Name	Sequence	Mass (g/mol)	Cys site
cys-iiaa	C G G D I A Q A I K Q I A E A I Q K I A G G Y	2,361	N
iiaa-cys	Y G G D I A Q A I K Q I A E A I Q K I A G G C G	2,418	C
cys-vlaa	C G G D L A Q A V K Q L A E A V Q K L A G G Y	2,333	N
vlaa-cys	Y G G D L A Q A V K Q L A E A V Q K L A G G C G	2,390	C
cys-liaa	C G G D I A Q A L K Q I A E A L Q K I A G G Y	2,361	N
liaa-cys	Y G G D I A Q A L K Q I A E A L Q K I A G G C G	2,418	C

glycine residue after the cysteine so that each member of an adeg-cys+cys-adeg pair had a different molecular weight, as shown in Table 2. Thus, after disulfide bond formation, each species present in solution would be distinguished by its molecular weight using mass spectrometry.

Figure 7 shows the strategy used in the cross-linking experiment. If the adeg peptide of interest forms only parallel, four-helix bundles then, after disulfide cross-linking, there would only be two molecular weight species present in solution. The two molecular weight species would correspond to two helices having a parallel orientation with a disulfide bond between two N-terminal cysteines (denoted NN) and also between two C-terminal cysteines (denoted CC). However, if the adeg peptide of interest forms only

antiparallel four-helix bundles, three molecular weight species would be detected corresponding to the NN and CC species mentioned above, as well as two antiparallel helices with a disulfide link between an N-terminal and a C-terminal cysteine (denoted NC).

Table 3 shows the results of mass spectrometry performed after the disulfide cross-linking of the peptides cys-iiaa + iiaa-cys, cys-vlaa + vlaa-cys, and cys-liaa + liaa-cys. In all cases, NC species were detected, which is consistent with antiparallel alignment. In addition, monomers were detected in the case of liaa (see Table 3), which is consistent with pentamer formation. In a five-helix bundle, two disulfide-bonded pairs would provide four helices, while the fifth helix would be excluded from a disulfide pair and remain monomeric.

To determine whether disulfide bond formation was compatible with the tetrameric structure of vlaa, we performed sedimentation equilibrium analysis on the disulfide-bonded mixture of cys-vlaa + vlaa-cys. We found that in 50 mM CH₃COONH₄ the data did not fit to a model for a single ideal species and was best described by an association model for a monomer–dimer–tetramer equilibrium. In this case, the monomer corresponds to the disulfide-linked species: NN, CC, and NC, which are shown in Table 3; these species are likely to be unfolded. The dimer corresponds to noncovalent association of two monomers described above and is likely to possess four-helix bundle structure. The tetramer is likely to be comprised of two four-helix bundle structures linked by disulfide bonds. In buffer containing 20 mM CH₃COONH₄ and 4 M Gdn-HCl, sedimentation analysis indicated that the peptides formed a monomer–dimer equilibrium mixture. Therefore, under these conditions the unfolded NN, CC, and NC species (monomer) were in equilibrium with four-helix bundle structures (dimer), and the disulfide-linked pair of four-helix bundles (tetramer) was absent.

Fig. 7. Strategy used in the assessment of the parallel vs. antiparallel alignment of helices in the adeg oligomers. M1 represents the molecular weight of the cys-adeg peptide, and M2 represents the molecular weight of the adeg-cys peptide. If the alignment of the adeg oligomer is (A) parallel, only two molecular weight species will be detected (M1 + M1 and M2 + M2), whereas if the alignment is (B) antiparallel, three molecular weight species will be detected (M1 + M1, M2 + M2, and M1 + M2) (Harbury et al., 1993).

Table 3. Assessing parallel vs. antiparallel alignment of adeg helical bundles

Peptide mixture	Mass of species detected by ES-MS (g/mol)
cys-iiaa + iiaa-cys	4,718 (NN), ^a 4,774 (NC), ^b 4,831 (CC) ^c
cys-vlaa + vlaa-cys	4,662 (NN), 4,718 (NC), 4,776 (CC)
cys-liaa + liaa-cys	4,717 (NN), 4,775 (NC), 4,832 (CC), 2,360 (N), 2,416 (C)

^aNN, parallel orientation with disulfide link between two N-termini.

^bNC, antiparallel orientation with disulfide link between N- and C-termini.

^cCC, parallel orientation with disulfide link between two C-termini.

Therefore, disulfide bonded mixtures of cys-vlaa + vlaa-cys are capable of forming antiparallel four-helix bundles, along with other oligomeric states; however, the antiparallel four-helix bundle state is the most stable thermodynamic state.

Amide proton exchange kinetics of vlaa

Another method for assessing conformational specificity in protein design studies is the measurement of amide proton exchange (Lumb & Kim, 1995). Under conditions where the EX2 amide proton exchange mechanism dominates, NH groups in folded structures with high conformational specificity exchange at rates that are significantly slower than those in unfolded states. The expected exchange rate for NH groups in an unfolded state, defined as the intrinsic exchange rate, can be calculated from model compound data (Bai et al., 1993). The high conformational specificity of the designed coiled coil ACID-p1/BASE-p1 was demonstrated by amide proton exchange measurements, which revealed three NH groups that exchange 10^5 -fold slower than the intrinsic exchange rate (Lumb & Kim, 1995). Amide proton exchange measurements have also established the high conformational specificity of the de novo designed peptide α_3 D, which contains slowly exchanging amide groups that exchange between 10^2 – 10^5 -fold slower than the intrinsic exchange rates (Walsh et al., 1999).

We examined the amide proton exchange rates of vlaa because it showed the highest degree of conformational specificity among the adeg peptides. In particular, vlaa had the lowest ANS binding characteristics and formed soluble tetramers with stable helical structure. The exchange kinetics of vlaa were too fast to measure at pH 7, but at pH 2.26 the exchange was slow enough to make accurate measurements. Sedimentation equilibrium ultracentrifugation of the pH 2.26 NMR sample indicated that under the NMR conditions (1 mM peptide concentration), vlaa remained predominantly tetrameric however a small amount ($\sim 10\%$) of a much larger aggregate was also present (data not shown). The pH dependence of the exchange kinetics indicated that the NH groups of vlaa exchanged by the EX2 exchange mechanism (Englander & Kallenbach, 1984). At pH 2.26, the simplest model that best described the exchange kinetics was a double exponential model in which there was one fast and one slow exchanging class of NH groups (Fig. 8). The rate constants and fractional concentrations for the fast- and slow-exchanging NH groups were $3.70 \times 10^{-4} \text{ s}^{-1}$ and $3.69 \times 10^{-5} \text{ s}^{-1}$, respectively, and 40 and 60%, respectively. The intrinsic exchange rates of the individual NH groups of vlaa, calculated using the method of Bai et al. (1993), ranged between 2.2×10^{-4} to 5.78×10^{-4} (mean value = $3.27 \times 10^{-4} \text{ s}^{-1}$). The rate constants for the NH groups in the fast-exchanging class corresponded closely to the intrinsic exchange rates, suggesting that these groups are unprotected. Conversely, the rate constant for the NH groups in the slow-exchanging class was 10-fold slower than the intrinsic rates. Thus, the NH groups in the slow-exchanging class show protection similar to that of a molten globule (Hughson et al., 1990), rather than a native protein. One plausible explanation for the structural origins of the fast- and slow-exchanging class of protons is that the fast-exchanging protons originate from unstructured or nonhydrogen bonded NH groups, and the NH groups that are hydrogen bonded within the helix make up the slow exchanging class. The vlaa peptide contains 21 NH groups. If we assume that the helix ends at the C-terminal -Gly-Gly-Tyr sequence, then 8 out of the 21 (or 38%) NH groups would not be hydrogen bonded within the helix. This percentage is very close to

Fig. 8. Amide proton exchange kinetics of vlaa. Amide proton exchange was monitored by ^1H -NMR using 1 mM total peptide concentration at pH 2.26, 5 °C. Fractional proton occupancy is determined as described in Materials and methods. The decay in fractional proton occupancy was fit to the equation: fractional occupancy = $A \exp(-k_1 t) + B \exp(-k_2 t)$. The best fit was obtained with the values: $A = 0.412$, $B = 0.608$, $k_1 = 3.70 \times 10^{-4} \text{ s}^{-1}$, $k_2 = 3.69 \times 10^{-5} \text{ s}^{-1}$.

the fractional concentration (40%) of the fast-exchanging class of NH groups, thus reinforcing the proposal that the fast-exchanging class is comprised of NH groups that are not hydrogen bonded.

Discussion

In a system designed to study assemblies of short helices, in which the potential for interhelical polar interactions has been minimized, we used a unique methodology to differentiate specific oligomers from structural states with low conformational specificity. We observed that subtle changes in the configuration of the nonpolar interactions resulted in a large variation in the extent of conformational specificity. In particular, permutations of Leu, Ile, and Val in the a and d heptad positions of the adeg consensus sequence caused the formation of the following structures: helical tetramers and pentamers, soluble and insoluble helical aggregates, insoluble unstructured aggregates, and soluble unstructured monomers. Both liaa and vlaa formed helical tetramers, and liaa formed helical pentamers. The stability of the specific oligomers was assessed by the concentration dependence of their helix content, and these data indicated that the rank order of stability was liaa > iiaa > vlaa. The ANS dye binding experiment indicated that the same rank order exists in terms of exposed hydrophobic surface area: liaa > iiaa > vlaa. A disulfide cross-linking experiment was used to assess the alignment of the helices in the tetramers and pentamers. This experiment indicated that antiparallel alignment of helices was present in the tetramers and pentamers. However, this method cannot eliminate the possibility that there is a mixture of both parallel and antiparallel helical bundles.

Interestingly, no specific hetero-oligomers were formed in the adeg series. One possible reason for this may be the asymmetry in the sequence, i.e., there are unequal numbers of a and d heptad positions in the consensus sequence. Alternatively, the lack of hetero-oligomer formation may be due to the absence of interactions between amino acids in the e and g heptad positions. Previously it has been shown that, in both naturally occurring and

designed coiled coils, interhelical electrostatic interactions between amino acids in the e and g heptad positions drive hetero-oligomer formation (O'Shea et al., 1989, 1993).

The method we developed for evaluating conformational specificity was capable of dealing with the large number of samples generated by the peptide series, and the array of possible structures formed. The procedure assessed the solubility, size, and secondary structure of peptide assemblies using an effective combination of common analytical techniques. This method may be adapted for use in other systems, for example, identifying soluble, folded proteins from expression libraries.

A number of other systematic studies have been performed on helical peptides with a heptad repeat in their sequences, whose a and d positions are occupied by Ile, Leu, or Val, exclusively. The properties of these systems are compared in Table 4. There are two major differences between our adeg peptides and the other peptide systems listed in Table 4. First, the identities of the e and g residues in the adeg peptides are Ala, whereas in the other systems the polar residues Glu, Gln, or Lys occupy e and g positions. Second, the length of the helical segments in the adeg series is significantly shorter (17 residues) than those in the other systems (29–35 residues). One common feature of all the systems listed is that peptides with Leu in the a and d positions do not possess conformational specificity. In contrast, the peptides in the adeg system, which formed specific oligomers, have oligomeric states that are one higher than the corresponding peptides in the other systems. For

example, in the adeg system iiaa and vlaa formed helical tetramers, while the analogous peptides in the other systems formed trimers and dimer/trimer mixtures, respectively. Furthermore, while liaa formed helical pentamers, the analogous peptides in the other systems formed helical tetramers. The small size of the adeg peptides could account for the higher oligomeric states observed in the adeg system compared to the coiled coil systems. The smaller hydrophobic surface area of the adeg peptides may require formation of higher order oligomers for stability. Another difference between the adeg system and the other systems listed in Table 4 (except coil-Ser) is that the adeg oligomers have an antiparallel alignment, whereas the helical assemblies in the other systems are parallel. This alignment difference may be a result of the shorter chain length of the adeg peptides. While there are numerous examples of antiparallel assemblies of short helices (<23 residues) (Schafmeister et al., 1993; Taylor et al., 1996; Hill & DeGrado, 1998; Prive et al., 1999), we are unaware of any examples of parallel assemblies of short helices less than 23 residues in length (Lumb et al., 1994).

Our finding that liaa forms pentameric helical bundles is noteworthy because this is a relatively uncommon structure. Designed five helix bundles include PERI COIL-1, which is an amphiphilic helical peptide containing periodic prolines (Kitakuni et al., 1994), and a "peptoid" pentamer, which is a polymer of N-substituted glycines (Armand et al., 1998). Naturally occurring five helix bundles include cartilage oligomeric matrix protein (COMP) (Malash-

Table 4. Comparison of the specificity patterns of the GCN4 analogs and the adeg peptides

System	Peptide name	a,d,e,g Residues	Oligomerization state in solution	Helix alignment
adeg	vlaa	V,L,A,A	Tetramer	Antiparallel
Peptides	iiaa	I,I,A,A	Tetramer	Antiparallel
Helix = 17 aa	liaa	L,I,A,A	Pentamer	Antiparallel
	ilaa	I,L,A,A	Soluble helical aggregate	—
	llaa	L,L,A,A	Insoluble helical aggregate	—
	viaa	V,I,A,A	Insoluble unstructured aggregate	—
	ivaa	I,V,A,A	Insoluble unstructured aggregate	—
	lvaa	L,V,A,A	Soluble unstructured monomer	—
	vvaa	V,V,A,A	Soluble unstructured monomer	—
GCN4	p-VL	V,L,E/K,E/K	Nonspecific	—
Analog ^a	p-II	I,I,E/K,E/K	Trimer	Parallel
Helix = 33 aa	p-LI	L,I,E/K,E/K	Tetramer	Parallel
	p-IL	I,L,E/K,E/K	Dimer	Parallel
	p-LL	L,L,E/K,E/K	Nonspecific	—
	p-VI	V,I,E/K,E/K	Nonspecific	—
	p-LV	L,V,E/K,E/K	Nonspecific	—
ABC ^b	ABC	I,I,E/K,E/K	Trimer	Parallel
Helix = 34 aa				
TM analog ^c	Nr	V,L,Q,Q	Dimer	Parallel
Helix = 35 aa				
Coil-V _a L _d ^d	Coil-V _a L _d	V,L,E,K	Trimer	Parallel
Helix = 29 aa				
Coil-Ser ^e	Coil-Ser	L,L,E,K	Monomer-dimer-trimer	Antiparallel
Helix = 29 aa				

^aHarbury et al. (1993).

^bNautiyal and Alber (1999).

^cKohn et al. (1995).

^dOgihara et al. (1997).

^eLovejoy et al. (1993).

kevich et al., 1996), phospholamban (Simmerman et al., 1996), acetylcholine receptor (Opella et al., 1999), and shiga-like toxin (Stein et al., 1992). One feature of all these five helix bundles is that they possess a central pore. The peptide liaa may form a pore, as well. As mentioned earlier, it was observed in the ANS dye binding experiment that liaa showed a large increase in ANS fluorescence (see Fig. 2D). Moreover, we observed that addition of benzene to saturation (18 mM final concentration) to the liaa + ANS sample caused a 30% decrease in the ANS fluorescence; the ANS fluorescence of all other adeg peptides did not decrease upon adding benzene (data not shown). It is possible, therefore, that the benzene was bound by the liaa oligomer in a central cavity.

Conclusions

The extensive work done on GCN4 analogs (O'Shea et al., 1991; Lumb & Kim, 1995; Gonzalez et al., 1996) and from de novo designed helical bundles (Handel et al., 1993; Hill & DeGrado, 1998) has led to a working definition for structural uniqueness based on the following criteria: cooperative thermal unfolding, dispersion in NMR spectra, a high level of protection from amide proton exchange, the existence of a single oligomeric state, and low ANS binding. Protein structures that do not meet all the above criteria are not considered to be structurally unique and are designated as nonspecific. Moreover, the above work has led to the hypothesis that hydrophobic interactions contribute stability, but not necessarily specificity to protein structure. The present work used a system in which interhelical polar side-chain interactions have been minimized, and buried polar interactions are absent; therefore, effects on specificity can be solely attributed to nonpolar interactions. We found that conservative sequence changes entailing either a rearrangement or deletion of methyl groups produced a distribution of states that differ in conformational specificity, although they are not structurally unique. At one end of this distribution, we found hydrophobic interface sequences that yielded peptides with extremely low conformational specificity in that they formed helical aggregates that precipitated. Adjacent in this distribution were polymeric assemblies of helices with hydrodynamic radii of 20 nm. At the other end of the distribution, we found particular sequences yielding peptides that possessed a relatively high degree of conformational specificity, in that they formed unique tetrameric and pentameric states. However, amide proton exchange indicated that vlaa, the adeg peptide with the greatest conformational specificity, still lacked structural uniqueness. Our results indicate that nonpolar interactions can make a significant contribution to conformational specificity, albeit the contribution appears to be less than that of buried polar interactions. It appears that a quantitative method is required to factorize the contributions of different interactions to conformational specificity.

Materials and methods

Peptide synthesis and purification

The peptides were synthesized by the solid phase method on a 9050 Plus PepSynthesizer (Perseptive Biosystems, Framingham, Massachusetts) as peptide amides. Fmoc/dimethylformamide chemistry was used with the Fmoc-PAL-PEG-PS resin. The deprotection step (removal of the Fmoc group) was performed using 20% piperidine in dimethylformamide and active ester coupling reac-

tions were performed using the coupling reagent HATU. After synthesis and final deprotection step, the N-terminal α -amino group was acetylated using 0.5 M acetic anhydride and 0.5 M pyridine. The peptides were cleaved from the resin in 95% trifluoroacetic acid, 5% ethanedithiol, and 2% triisopropylsilane for 4 h. The cleaved peptides were separated from the resin by filtration, precipitated in diethyl ether, and washed twice in diethyl ether. After washing, the peptides were dissolved in water and purified by reversed-phase HPLC using a C18 radial pak cartridge on the Waters HPLC system. A wavelength of 230 nm was used for detection. Peptide identity was confirmed by electrospray mass spectrometry (ES-MS).

Peptide concentration determination

Because all peptides contained one tyrosine residue in their sequence, their concentration could be determined by measuring tyrosine absorbance at 275 nm. Aliquots of stock solutions were dissolved in 6.5 M guanidine hydrochloride, absorbance at 275 nm was measured using a Milton Roy Spectronic 3000 Array UV Spectrophotometer, and the concentration was determined using Beer's Law and an extinction coefficient of $\epsilon = 1,450 \text{ M}^{-1} \text{ cm}^{-1}$ (Brandts & Kaplan, 1973).

CD spectroscopy

CD measurements were performed on an Aviv 62A DS circular dichroism spectrometer. All CD measurements are reported in $\text{deg cm}^2 \text{ dmol}^{-1}$. Spectra were obtained using a 1 mm quartz cuvette at 25 °C in 10 mM Na_2HPO_4 , 120 mM NaCl, pH 7. Peptide concentrations were in the range 69–76 μM , and the wavelength range was 195–260 nm. Concentration-dependence experiments and the CD screening experiment were performed at a wavelength of 222 nm using a 1 cm quartz cuvette in 10 mM Na_2HPO_4 , 120 mM NaCl, pH 7 at 25 °C. A total peptide concentration of 50 μM was used for the CD screening experiment.

Fluorescence spectroscopy

Steady-state fluorescence was measured at room temperature using a Photon Technology International QM-1 fluorescence spectrophotometer equipped with excitation intensity correction and a magnetic stirrer. Emission spectra of the ANS dye were collected from 450 to 600 nm using an excitation wavelength of 372 nm, at 1 s/nm, and a bandpass of 4 nm for excitation and emission. Samples consisted of 50 μM peptide and 50 μM ANS in 10 mM Na_2HPO_4 , 120 mM NaCl, pH 7 at 25 °C. Another set of measurements was also made on samples containing 50 μM peptide, 50 μM ANS, and saturated benzene in 10 mM Na_2HPO_4 , 120 mM NaCl, pH 7 at 25 °C.

Solubility measurements

Solubility of the peptide mixtures was measured by monitoring UV absorbance before and after centrifugation. Each sample was prepared in a total volume of 500 μL consisting of 50 μM total peptide in 10 mM Na_2HPO_4 , 120 mM NaCl, pH 7 at 25 °C. To remove insoluble aggregates, the samples were centrifuged in an IEC MicroMax microfuge for 30 min at 13,200 rpm; the supernatant from each sample was then transferred to a new tube. Because each peptide contained one tyrosine in its sequence, the

absorbance of the supernatant was measured at 275 nm (A_{275}), using a Milton Roy Spectronic 3000 Array UV Spectrophotometer. A 50 μ M solution of soluble peptide containing a single tyrosine should have $A_{275} = 0.0695$. An absorbance value less than 0.0695 indicated that the peptide or peptide mixture formed insoluble aggregates that sedimented during centrifugation. Samples where $A_{275} = 0.060$ – 0.075 were considered 100% soluble; samples where $A_{275} = 0.025$ – 0.045 were 50% soluble; and samples where $A_{275} < 0.025$ were 0% soluble.

Light scattering

Light scattering experiments were performed on a DynaPro Dynamic Light Scattering Instrument with DYNAMICS software from Protein Solution (Charlottesville, Virginia). Each sample was prepared in a total volume of 500 μ L consisting of 50 μ M total peptide in 10 mM Na_2HPO_4 , 120 mM NaCl, pH 7 at 25 °C. To eliminate the presence of dust and insoluble aggregates, the samples were centrifuged in an IEC MicroMax microfuge for 30 min at 13,200 rpm; the supernatant from each sample was then transferred to a new tube. Measurements of scattered light intensity, or counts, were made using a sample volume of 20 μ L in a cuvette. The average scattering intensity of ddH_2O and buffer (10 mM Na_2HPO_4 , 120 mM NaCl, pH 7) was 5×10^3 counts/s over a period of 5 min with minimal fluctuations; this value was used as a control, because neither ddH_2O nor buffer would contain any detectable particles.

Sedimentation equilibrium ultracentrifugation

The molecular weights of the adeg oligomers and cys-vlaa + vlaa-cys peptides were determined by sedimentation equilibrium on a Beckman Optima XL-I analytical ultracentrifuge. Experiments were performed on the peptides vlaa, liaa, and iiaa in a six-sector cell with quartz windows in buffer containing 10 mM Na_2HPO_4 , 120 mM NaCl, pH 7 at 20 °C with total peptide concentrations of 50, 75, and 100 μ M. The SedNterp software was used to calculate the solvent density and the partial specific volumes of the peptides. A value of 1.00454 g/mL was calculated for the solvent density, and the partial specific volumes were calculated to be 0.7599, 0.7541, and 0.7599 mL/g, for iiaa, vlaa, and liaa, respectively. Measurements were made every 0.001 cm using 50 replicates. For the experiments using liaa and vlaa, data were collected after 20 h at a wavelength of 275 nm, using rotor speeds of 26,500, 29,000, and 31,000 rpm. Data fitting and analysis were performed with Microcal Origin 4.1 using a self-association model. Global data fits were obtained by fitting nine sets of data simultaneously to an equation for a single ideal species. The experiment using iiaa was performed at a wavelength of 230.5 nm using a rotor speed of 25,000 rpm, and data were collected after 20 h; a global data fit was performed by fitting three sets of data simultaneously to an equation for a single ideal species.

In addition, an experiment was performed on cys-vlaa + vlaa-cys in a six-sector cell with quartz windows in buffer containing 50 mM $\text{CH}_3\text{COONH}_4$ with total peptide concentrations of 30, 40, and 50 μ M. Using the SedNterp software, the solvent density was calculated to be 0.99815 g/mL, and the partial specific volume of cys-vlaa + vlaa-cys was 0.7415 mL/g. Measurements were made every 0.001 cm using 50 replicates; data were collected after 20 h at a wavelength of 230.5 nm using rotor speeds of 33,000, 37,000, and 40,000 rpm. Global data fits were obtained by fitting nine sets

of data to a monomer–dimer–tetramer equilibrium using Microcal Origin 4.1. Another experiment was performed with cys-vlaa + vlaa-cys in slightly denaturing conditions using buffer containing 20 mM $\text{CH}_3\text{COONH}_4$ and 4 M Gdn-HCl, with total peptide concentrations of 20, 17, and 14 μ M. A solvent density of 1.09632 g/mL was obtained from the SedNterp software. Data collection and rotor speeds were the same as above. Global data fits were obtained by simultaneously fitting nine sets of data to a monomer–dimer equilibrium using Microcal Origin 4.1.

Assessment of parallel vs. antiparallel alignment of helices in helical bundles through disulfide cross-linking

Each sample consisted of two cysteine-containing peptides: one with an N-terminal cysteine and the other with a C-terminal cysteine. In each sample, the two peptides had different molecular weights (see Table 2), so that after cross-linking the different species were distinguished by their molecular weights using mass spectrometry. The samples were prepared in a total volume of 8 mL consisting of 50 μ M peptide in 6.5 M Gdn-HCl, 1% β -mercaptoethanol, pH 8. All pH titrations were performed using NH_4OH . On day 1, the sample was dialyzed overnight in a dialysis buffer that contained distilled, deionized water and 1% β -mercaptoethanol, pH 8. On day 2, the dialysis buffer was changed to distilled, deionized water, pH 8, to remove the β -mercaptoethanol and allow disulfide bond formation. The dialysis buffer was changed three times at 3 h intervals. On day 3, the sample was lyophilized. Once dry, the sample was dissolved in a buffer containing 50% acetonitrile, 0.1% acetic acid, and 2 mM $\text{CH}_3\text{COONH}_4$ to a final concentration of 0.5 mM. The sample was then analyzed by ES-MS.

As a control, cys-vlaa + vlaa-cys was prepared as above, for analysis using sedimentation equilibrium ultracentrifugation. The final equilibrium conditions were in dialysis buffer containing 50 mM $\text{CH}_3\text{COONH}_4$ and a total peptide concentration of 50 μ M.

Amide proton exchange rate measurements

Amide proton exchange was measured by ^1H -NMR spectroscopy. An aqueous solution of vlaa (1 mM, 700 μ L) was adjusted to pH 2.26 and lyophilized. At $t = 0$ min, vlaa was dissolved in 700 μ L of D_2O containing 5 mM NaH_2PO_4 , pH* 2.26 (the glass electrode reading at room temperature, uncorrected for isotope effects). Multiple one-dimensional proton spectra were acquired using a Varian 500 Unity Plus spectrometer at a proton frequency of 500.053 MHz. Data were collected using a 7,000.4 Hz spectral width and a 90° pulse. Presaturation was used to suppress residual HDO signal. The peaks in the amide region were integrated and normalized using one of the nonexchanging tyrosine side-chain resonance peaks. The proton occupancy was determined by dividing the normalized amide peak areas by the extrapolated area at $t = 0$ min.

Acknowledgments

This work was supported by a grant from the Medical Research Council of Canada. C.B. acknowledges financial support from the Ontario Student Opportunity Transfer Fund. We thank Robert Fairman and Alan Davidson for valuable suggestions, Gene Merutka, and Thomas Leung, for experimental contributions early in this work, and Xiao-Fei Qi, Sandy Go, Jackson Huang, and JoAnne McLaurin for technical assistance.

References

- Armand P, Kirshenbaum K, Goldsmith RA, Farr-Jones S, Barron AE, Truong KT, Dill KA, Mierke DF, Cohen FE, Zuckermann RN, Bradley EK. 1998. NMR determination of the major solution conformation of a peptoid pentamer with chiral side chains. *Proc Natl Acad Sci USA* 95:4309–4314.
- Bai Y, Milne JS, Mayne L, Englander SW. 1993. Primary structure effects on peptide group hydrogen exchange. *Proteins Struct Funct Genet* 17:75–86.
- Brandts JF, Kaplan LJ. 1973. Derivative spectroscopy applied to tyrosyl chromophores. Studies on ribonuclease, lima bean inhibitors, insulin, and pancreatic trypsin inhibitor. *Biochemistry* 12:2011–2024.
- Chakrabartty A, Baldwin RL. 1995. Stability of alpha-helices. *Adv Protein Chem* 46:141–176.
- Chakrabartty A, Kortemme T, Padmanabhan S, Baldwin RL. 1993. Aromatic side-chain contribution to far-ultraviolet circular dichroism of helical peptides and its effect on measurement of helix propensities. *Biochemistry* 32:5560–5565.
- Cohen C, Parry DA. 1990. Alpha-helical coiled coils and bundles: How to design an alpha-helical protein. *Proteins Struct Funct Genet* 7:1–15.
- DeGrado WF, Lear JD. 1985. Induction of peptide conformation at apolar/water interfaces. 1. A study with model peptides of defined hydrophobic periodicity. *J Am Chem Soc* 107:7684–7689.
- Dill KA. 1990. Dominant forces in protein folding. *Biochemistry* 29:7133–7155.
- Dill KA, Bromberg S, Yue K, Fiebig KM, Yee DP, Thomas PD, Chan HS. 1995. Principles of protein folding—A perspective from simple exact models. *Protein Sci* 4:561–602.
- Englander SW, Kallenbach NR. 1984. Hydrogen exchange and structural dynamics of proteins and nucleic acids. *Q Rev Biophys* 16:521–655.
- Gonzalez L Jr, Woolfson DN, Alber T. 1996. Buried polar residues and structural specificity in the GCN4 leucine zipper. *Nat Struct Biol* 3:1011–1018.
- Handel TM, Williams SA, DeGrado WF. 1993. Metal ion-dependent modulation of the dynamics of a designed protein. *Science* 261:879–885.
- Harbury PB, Zhang T, Kim PS, Alber T. 1993. A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. *Science* 262:1401–1407.
- Hill BR, DeGrado WF. 1998. Solution structure of alpha2D, a natively like de novo designed protein. *J Am Chem Soc* 120:1138–1145.
- Ho SP, DeGrado WF. 1987. Design of a 4-helix bundle protein: Synthesis of peptides which self-associate into a helical protein. *J Am Chem Soc* 109:6751–6758.
- Hughson FM, Wright PE, Baldwin RL. 1990. Structural characterization of a partly folded apomyoglobin intermediate. *Science* 249:1544–1548.
- Kitakuni E, Kuroda Y, Oobatake M, Tanaka T, Nakamura H. 1994. Thermodynamic characterization of an artificially designed amphiphilic alpha-helical peptide containing periodic prolines: Observations of high thermal stability and cold denaturation. *Protein Sci* 3:831–837.
- Kohn WD, Kay CM, Hodges RS. 1995. Protein destabilization by electrostatic repulsions in the two-stranded alpha-helical coiled-coil/leucine zipper. *Protein Sci* 4:237–250.
- Kuwajima K. 1989. The molten globule state as a clue for understanding the folding and cooperativity of globular-protein structure. *Proteins Struct Funct Genet* 6:87–103.
- Lovejoy B, Choe S, Cascio D, McRorie DK, DeGrado WF, Eisenberg D. 1993. Crystal structure of a synthetic triple-stranded alpha-helical bundle. *Science* 259:1288–1293.
- Lumb KJ, Carr CM, Kim PS. 1994. Subdomain folding of the coiled coil leucine zipper from the bZIP transcriptional activator GCN4. *Biochemistry* 33:7361–7367.
- Lumb KJ, Kim PS. 1995. A buried polar interaction imparts structural uniqueness in a designed heterodimeric coiled coil. *Biochemistry* 34:8642–8648.
- Malashkevich VN, Kammerer RA, Efimov VP, Schulthess T, Engel J. 1996. The crystal structure of a five-stranded coiled coil in COMP: A prototype ion channel? *Science* 274:761–765.
- Marqusee S, Baldwin RL. 1987. Helix stabilization by Glu...Lys+ salt bridges in short peptides of de novo design. *Proc Natl Acad Sci USA* 84:8898–8902.
- Monera OD, Zhou NE, Kay CM, Hodges RS. 1993. Comparison of antiparallel and parallel two-stranded alpha-helical coiled-coils. Design, synthesis, and characterization. *J Biol Chem* 268:19218–19227.
- Munson M, Balasubramanian S, Fleming KG, Nagi AD, O'Brien R, Sturtevant JM, Regan L. 1996. What makes a protein a protein? Hydrophobic core designs that specify stability and structural properties. *Protein Sci* 5:1584–1593.
- Nautiyal S, Alber T. 1999. Crystal structure of a designed, thermostable, heterotrimeric coiled coil. *Protein Sci* 8:84–90.
- Nautiyal S, Woolfson DN, King DS, Alber T. 1995. A designed heterotrimeric coiled coil. *Biochemistry* 34:11545–11651.
- Oakley MG, Kim PS. 1997. Protein dissection of the antiparallel coiled coil from *Escherichia coli* seryl tRNA synthetase. *Biochemistry* 36:2544–2549.
- Ogihara NL, Weiss MS, DeGrado WF, Eisenberg D. 1997. The crystal structure of the designed trimeric coiled coil coil-V_aL_d: Implications for engineering crystals and supramolecular assemblies. *Protein Sci* 6:80–88.
- Opella SJ, Marassi FM, Gesell JJ, Valente AP, Kim Y, Oblatt-Montal M, Montal M. 1999. Structures of the M2 channel-lining segments from nicotinic acetylcholine and NMDA receptors by NMR spectroscopy. *Nat Struct Biol* 6:374–379.
- O'Shea EK, Klemm JD, Kim PS, Alber T. 1991. X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. *Science* 254:539–544.
- O'Shea EK, Lumb KJ, Kim PS. 1993. Peptide "Velcro": Design of a heterodimeric coiled coil. *Curr Biol* 3:658–667.
- O'Shea EK, Rutkowski R, Stafford WF 3d, Kim PS. 1989. Preferential heterodimer formation by isolated leucine zippers from *fos* and *jun*. *Science* 245:646–648.
- Prive GG, Anderson DH, Wesson L, Cascio D, Eisenberg D. 1999. Packed protein bilayers in the 0.90 Å resolution structure of a designed alpha helical bundle. *Protein Sci* 8:1400–1409.
- Richardson JS, Richardson DC. 1988. Amino acid preferences for specific locations at the ends of alpha helices. *Science* 240:1648–1652.
- Roy S, Ratnaswamy G, Boice JA, Fairman R, McLendon G, Hecht MH. 1997. A protein designed by binary patterning of polar and nonpolar amino acids displays native-like properties. *J Am Chem Soc* 119:5302–5306.
- Schafmeister CE, Miercke LJ, Stroud RM. 1993. Structure at 2.5 Å of a designed peptide that maintains solubility of membrane proteins. *Science* 262:734–738.
- Shoemaker KR, Kim PS, York EJ, Stewart JM, Baldwin RL. 1987. Tests of the helix dipole model for stabilization of alpha-helices. *Nature* 326:563–567.
- Simmerman HK, Kobayashi YM, Autry JM, Jones LR. 1996. A leucine zipper stabilizes the pentameric membrane domain of phospholamban and forms a coiled-coil pore structure. *J Biol Chem* 271:5941–5946.
- Stein PE, Boodhoo A, Tyrrell GJ, Brunton JL, Read RJ. 1992. Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E. coli*. *Nature* 355:748–750.
- Taylor KS, Lou MZ, Chin TM, Yang NC, Garavito RM. 1996. A novel, multi-layer structure of a helical peptide. *Protein Sci* 5:414–421.
- Walsh STR, Cheng H, Bryson JW, Roder H, DeGrado WF. 1999. Solution structure and dynamics of a de novo designed three-helix bundle protein. *Proc Natl Acad Sci USA* 96:5486–5491.
- Zeng X, Zhu H, Lashuel HA, Hu JC. 1997. Oligomerization properties of GCN4 Leucine zipper e and g position mutants. *Protein Sci* 6:2218–2226.