Design of two-stranded and three-stranded coiled-coil peptides

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SUMMARY

The structural features required for the formation of two- versus three-stranded coiled coils have been explored using *de novo* protein design. Peptides with leucine at the 'a' and 'd' positions of a coiled-coil (general sequence: $Leu_a\ Xaa_b\ Xaa_c\ Leu_d\ Glu_e\ Xaa_f\ Lys_g$) exist in a non-cooperative equilibrium between unstructured monomers and helical dimers and helical trimers. Substituting valine into each 'a' position produces peptides which still form trimers at high concentrations, whereas substitution of a single asparagine at the 'a' position of the third heptad yields a dimer.

During the course of this work, we also re-investigated a helical propensity scale derived using a series of coiled-coil peptides previously believed to exist in a monomer–dimer equilibrium (O'Neil & DeGrado 1990). Detailed analysis of the concentration dependence of ellipticity at 222 nm reveals that they exist in a non-cooperative monomer–dimer–trimer equilibrium. However, the concentration of trimer near the midpoint of the concentration-dependent transition is small, so the previously determined values of $\Delta\Delta G_\alpha$ using the approximate monomer–dimer scheme are indistinguishable from the values obtained employing the complete monomer–dimer–trimer equilibrium.

1. INTRODUCTION

The *de novo* design of globular proteins with predetermined structures and functions is a challenging goal that critically tests our understanding of the determinants of protein folding (DeGrado *et al.* 1991; Betz *et al.* 1993). In recent years, a variety of different proteins and peptides have been prepared including four-helix bundles (Handel *et al.* 1993; Choma *et al.* 1994), β-sandwich proteins (Quinn *et al.* 1994; Yan & Erickson 1994), and mixed α-β proteins (Beauregard *et al.* 1991; Tanaka *et al.* 1994). A simpler, but nevertheless challenging problem is the design of peptides that assemble into α-helical coiled-coil conformations (Graddis *et al.* 1993; O'Shea *et al.* 1993; Monera *et al.* 1994; Myszka & Chaiken 1994; Zhou *et al.* 1994;). The coiled coil is a simple structure which exhibits most of the characteristics of native proteins, including the formation of secondary structure stabilized by hydrophobic and electrostatic interactions. In coiled coils, two or more α-helices wrap around one another with a slight left-handed helical twist, forming a superhelix. Coiled coils exhibit a periodic primary structure which repeats every seven residues. Thus the design of a coiled-coil protein reduces to the problem of designing seven-residue peptide repeats which will lead to the formation of a given aggregation state.

In the past, the general structures and forces stabilizing coiled coils have been extensively studied (Crick 1953; Cohen & Parry 1990). Figures 1 and 2*a* illustrate structures for two-stranded and three-stranded coiled coils. In both cases, amino acid residues traditionally referred to as positions 'a' and 'd' project inward towards the superhelical axis. These residues are generally apolar and serve to stabilize the structure through hydrophobic interactions. The structures of two different classes of synthetic coiled coils have been determined. The coiled coil from GCN4 has been determined at high resolution, and found to form a two-stranded coiled coil that is very similar to the idealized structure shown in figure 1*a* (O'Shea *et al.* 1991). The sequence of this peptide consists of about four heptads. It contains Leu at each 'd' position and Val at each 'a' position with the exception of a single Asn at the 'a' position of the third heptad. The effects of substituting other residues into the 'a' and 'd' positions of all four heptads have been investigated. In this system, peptides with Leu at 'a' and Ile at 'd' form tetramers (Harbury *et al.* 1993), and peptides with Ile at all 'a' and 'd' positions form trimers (Harbury *et al.* 1994). These differences in association state have been rationalized in terms of the distinct packing preferences for Leu versus the β-branched amino acids Ile and Val.

In our own laboratory, we focused on a *de novo* designed peptide, originally intended to form a two-stranded coiled coil as a model system for determining

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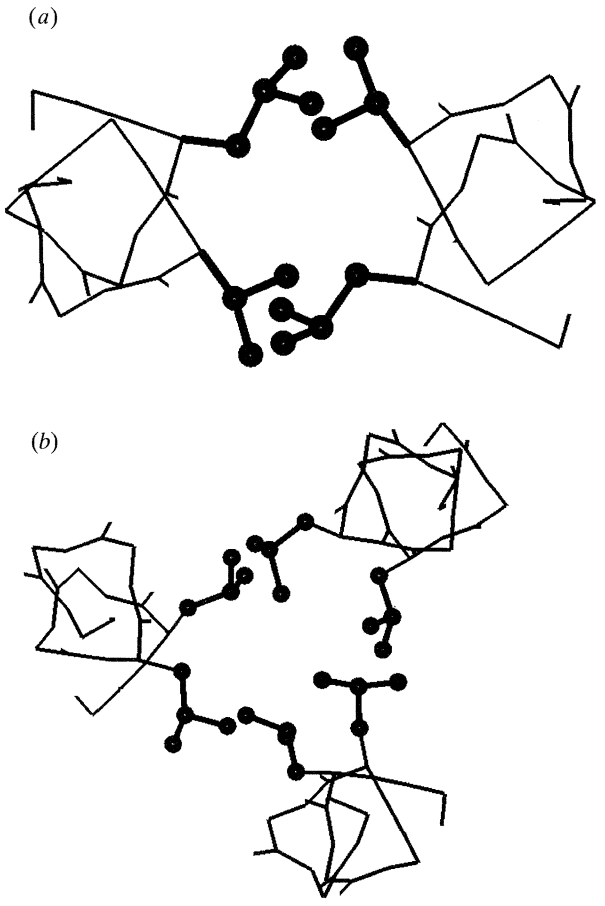


Figure 1. Hydrophobic core packing in coiled coils. 'a' and 'd' position side chains are shown as balls and sticks: (*a*) Parallel two-stranded coiled-coil from GCN4 (O'Shea *et al.* 1991); (*b*) Anti-parallel three-stranded coiled-coil from coil-Ser (Lovejoy *et al.* 1993).

helix propensities (O'Neil & DeGrado 1990). In an effort to determine an α-helical propensity scale, we wished to develop a system in which a peptide switches from being in the random-coil state to a fully α-helical state in a single transition that is thermodynamically linked to a two-state process, dimerization. A two-stranded coiled coil was designed that included a site for substituting 'guest' amino acids on the solvent-exposed face of the helix (see figure 2). The design of the helical dimer was based on the peptides of Hodges and co-workers (Lau *et al.* 1984). Following Hodges: the peptides contain Leu residues at the 'a' and 'd' positions, included to hydrophobically stabilize the structure, as well as interhelical electrostatic interactions between Glu and Lys residues on neighboring helices. The guest site is surrounded by neutral Ala residues to minimize the interactions between the side chain of the guest and those of the host. A set of 20 peptides was prepared with each of the commonly occurring amino acids substituted into the guest position. These peptides showed concentration-dependent circular dichroism (CD) spectra which could be analysed in terms of an equilibrium between random-coil monomers and α-helical dimers. The differences between the free energies of dimerization for the various peptides ($\Delta\Delta G_\alpha$) were dependent on the helix-forming character of the guest amino acid, which allowed the derivation of a new scale of intrinsic helical propensities (O'Neil & DeGrado 1990).

After completing determination of the helix propensities, the crystal structure of one of the peptides, coil-Ser (named for the amino acid at the guest position) was determined and found to be a trimer rather than the expected dimer (Lovejoy *et al.* 1993). As expected, the structure consists of a pair of parallel helices, with the 'host' site at the 'f' position fully exposed to solvent. However, a third helix docks against the dimer anti-parallel to the other pair (see figures 1*b* and 2*a*). Sedimentation equilibrium showed that the peptide formed trimers in solution at micromolar concentrations (Lovejoy *et al.* 1993). These findings stimulated a thorough re-examination of the thermodynamics of self-association of the Coil-Xaa family of peptides (see figure 2*b*). Here we show that the peptides exist in a non-cooperative monomer–dimer–trimer equilibrium in which the free energy of dimerization of two monomers is approximately equal to the free energy of adding a monomer to a dimer to form a trimer. This analysis allowed the calculation of a corrected helix thermodynamic scale ($\Delta\Delta G_\alpha$). Fortunately, the values of $\Delta\Delta G_\alpha$ are indistinguishable from the earlier values within the error of our measurements.

We have also investigated the structural features that cause the peptide to form trimers rather than dimers. Figure 2 illustrates the sequence coil-Ser as compared to the two-stranded coiled-coil of GCN4. Both have Leu residues at the 'd' position, but differ in the residues at the 'a' position, including the presence of Trp in the first heptad of Coil-Ser. This residue was included as a spectroscopic probe, and could conceivably direct the antiparallel orientation of the helices in the trimer by disfavouring an all-parallel topology. Models suggest that if the peptides adopt a parallel trimer, the bulky Trp residues bump into one another in an unfavourable interaction that is absent in the antiparallel structure. We therefore synthesized the peptide coil-L_aL_d, in which the Trp is changed to Leu resulting in a peptide with Leu in every 'a' and 'd' position. GCN4 contains Val residues at each of its 'a' positions so we also investigated changing all the Leu residues in the 'a' positions to Val in peptide coil-V_a-L_d. Additionally, we investigated the role of the Asn at an 'a' position in GCN4 through the synthesis of Asn_{16}-coil-V_a-L_d. Finally, in an effort to reconcile our results with earlier results by Hodges on his coiled-coil peptides, we prepared TM-43, that also contains Leu residues at positions 'a' and 'd'.

2. METHODS

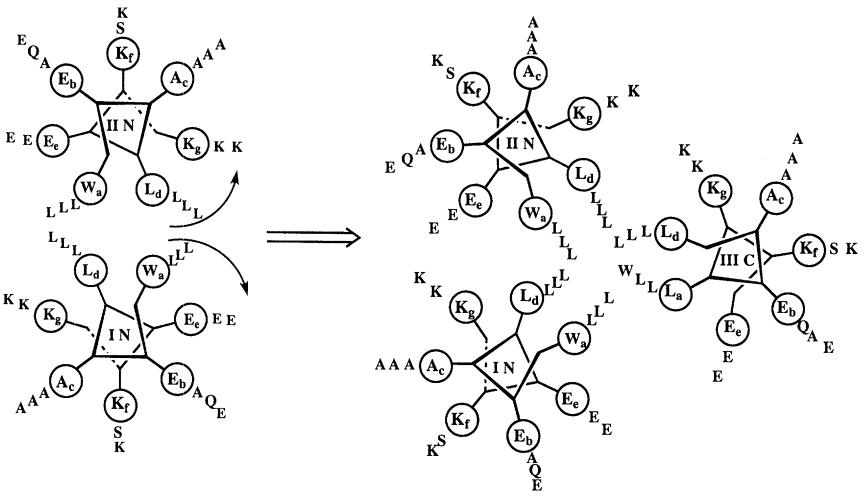
(*a*) *Peptides*

The peptides TM-43, Asn_{16}-coil-V_a-L_d, coil-V_a-L_d, and coil-L_a-L_d were prepared using Fmoc-protected amino acids and techniques described previously (Choma *et al.* 1994).

(*b*) *Sedimentation equilibrium*

Sedimentation equilibrium analysis was performed using a Beckman XLA analytical ultracentrifuge. The

(*a*)



(*b*)

peptide		heptad position				
	g	abcdefg	abcdefg	abcdefg	abcdefg	abcdefg
coil-Xaa	E	WEALEKK	LAALEXK	LQALEKK	LEALEHG	
coil-L_aL_d	E	LEALEKK	LAALESK	LQALEKK	LEALEHG	
coil-V_aL_d	E	VEALEKK	VAALESK	VQALEKK	VEALEHG	
Asn_{16}-coil-V_aL_d	E	VEALEKK	VAALESK	NQALEKK	VEALEHG	
TM-43	K	LEALEGK	LEALEGK	LEALEGK	LEALEGK	$(LEALEGK)_2$
GCN4	R	MKQLEDK	VEELLSK	NYHLENE	VARLKKL	VGER

Figure 2. (*a*) Helical wheel representations of the desired packing of a parallel two-stranded coiled-coil versus the observed anti-parallel three-stranded coiled-coil in coil-Ser. The structures are related by the conceptual rotation of the two parallel helices (indicated by arrows), followed by the packing of the third helix in an antiparallel orientation. (*b*) Amino acid sequences of the peptides discussed in this paper, the top row indicates position within the coiled-coil heptad.

conditions for measuring the sedimentation behaviour, and analysis of coil-Ser, coil-Ala, coil-Arg, coil-Trp, Asn_{16}-coil-V_a-L_d, coil-V_a-L_d, and coil-L_a-$L_d$have been published previously (Lovejoy *et al.* 1993). Initial peptide concentrations were 20 μM and 200 μM. The conditions for TM-43 were chosen to match those of Hodges and co-workers (Lau *et al.* 1984): 50 mm potassium phosphate, pH 7.0 or 2.5, containing either 0.1 M KCl or 1.1 M KCl. The samples were centrifuged at 25000, 40000, and 48000 rpm; equilibrium was determined when successive radial scans at the same speed were indistinguishable. Partial specific volumes were determined by the residue-weighted average method of Cohn and Edsall (Cohn & Edsall 1943; Harding *et al.* 1992), as well as the H_2O–D_2O difference method (Edelstein & Schachman 1973) and found to agree within 0.01 ml g^{-1}. Solution densities were estimated using solute concentration-dependent density tables in the CRC Handbook of Chemistry and Physics. The aggregation state was determined by monomer-*n*mer equilibria. Also, fitting the data as a single species yielded molecular masses consistent with these aggregation states. Data were analysed with the software package Igor Pro (WaveMetrics, Inc.).

(*c*) *Analysis of the concentration dependence of the* CD *spectra*

The data can be fit to an empirically derived Hill coefficient, which examines the cooperativity of the association according to equations (1) and (2):

$$K_H = [P_{mon}]^n/[P_n], \quad (1)$$

$$[P_T] = [P_{mon}]^n/(K_H + [P_{mon}]), \quad (2)$$

in which P_{mon}, P_n, and P_T are the concentrations of the monomer, *n*mer and total peptide, respectively, *n* is the Hill coefficient, and K_H is a dissociation constant. Equation (2) is solved numerically for $[P_{mon}]$ using the

root function of Mlab (Civilized Software), and inserted into equation (3):

$$[\theta_{222}] = [\theta_{n\text{mer}}]\,(f) = [\theta_{n\text{mer}}]\,(([P_{\text{mon}}]^n/K_{\text{H}})/P_{\text{T}}, \quad (3)$$

in which $[\theta_{n\text{mer}}]$ is the mean residue ellipticity of the associated form of the peptide and f is the fraction of the peptide in the associated form. The parameters, $[\theta_{n\text{mer}}]$, n, and K_{H} are then determined for each peptide with the nonlinear least squares method using Mlab. This method of analysis assumes that the ellipticity of the monomer is zero; and was confirmed by allowing the ellipticity of the monomer to be an additional adjustable parameter, in which case the determined value was consistently within 3000 deg cm^2 dmol^{-1} of zero.

Alternatively the data could fit a monomer–dimer–trimer equilibrium as described in equation (7) in Results. The mass balance equation is:

$$\begin{aligned}[P_{\text{T}}] &= \text{M} + 2\,[\text{D}] + 3\,[\text{T}] \\ &= [\text{M}] + 2\,[\text{M}]^2/K_1 + 3\,[\text{M}]^3/K_1K_2. \end{aligned} \quad (4)$$

The mean residue ellipticity at a given peptide concentration is given by:

$$[\theta_{222}] = ([\text{M}]\,[\theta_{\text{M}}] + 2\,[\text{D}]\,[\theta_{\text{D}}] + 3\,[\text{T}]\,[\theta_{\text{T}}])/[\text{P}_{\text{T}}]. \quad (5)$$

in which the mean residue ellipticity of the dimer, $[\theta_{\text{D}}]$, and the trimer, $[\theta_{\text{D}}]$, are assumed to be identical; and the ellipticity of the monomer, $[\theta_{\text{m}}]$, is assumed to be zero. The parameters $[\theta_{\text{D}}]$, $[\theta_{\text{T}}]$, $[K_1]$ and $[K_2]$ are then determined for each peptide by the nonlinear least squares method using Mlab. In several calculations, the ellipticity of the monomer was included as an additional adjustable parameter; the determined value of this parameter in each case was consistently within 3000 deg cm^2 dmol^{-1} of zero.

The data were also analysed according to equations (8) and (9) in Results. With this approach a given peptide is chosen as a reference peptide, and values of K_1 and K_2 are calculated from the concentration dependence of its CD spectrum. The data for each additional peptide are then calculated using the mass balance equation:

$$\begin{aligned}[P_{\text{T}}] &= [\text{M}] + 2\,[\text{D}] + 3\,[\text{T}] \\ &= [\text{M}] + 2\,[\text{M}]^2/K_1' + 3\,[\text{M}]^3/K_1'K_2', \end{aligned} \quad (6)$$

in which K_1' and K_2' are as defined in equations (8) and (9). Equation (6) is once again numerically solved for [M] and inserted into equation (5). Now, however, because the values of K_1 and K_2 are known, the only fitted parameters are $\Delta\Delta G_\alpha$ and the ellipticities of the dimers and trimers (assumed to be equal). Coil-Ser was chosen because we had an extensive set of data for this peptide with values of K_1 and K_2 of 8.1 ± 2.5 and 5.0 ± 1.3, respectively. The values of $\Delta\Delta G_\alpha$ were then calculated for each of the remaining peptides and were referenced to Gly.

3. RESULTS

(*a*) *Sedimentation equilibrium studies*

We had adequate quantities of four peptides previously used in the helix propensity experiments (O'Neil & DeGrado 1990) to determine their aggregation states by sedimentation equilibrium (see table 1). At neutral pH, room temperature, with no denaturants and peptide concentrations ranging from 20–200 μM, the peptides sediment as single, homogeneous species with molecular masses consistent with a trimeric aggregation state. These data are consistent with earlier studies that showed the peptides self-assembled into highly stable helical aggregates that only dissociate in the presence of 4–7 M urea (O'Neil & DeGrado 1990).

The peptides coil-V_a-L_d and coil-L_a-L_d also were also found to be trimeric under these conditions, and only the peptide Asn_{16}-coil-V_a-$L_d$was found to form dimers. At an initial loading concentration of 1 mM this peptide sedimented as a single species with a molecular mass close to that expected for a dimer (see table 1).

The trimeric association state observed for coil-L_a-L_d is in marked contrast with the dimeric state reported by Hodges and co-workers for the parent coiled-coil peptide (Lau *et al.* 1984). We therefore prepared the parent coiled-coil peptide, TM-43, and determined its aggregation state by sedimentation equilibrium (see figure 3) at initial loading concentrations of 60 μM or 600 μM, pH 7.0 or 2.5, and 0.1 M KCl or 1.1 M KCl. At pH 7.0 (either combination of peptide and salt concentration), the data clearly indicate that this peptide forms trimers (see table 2). At pH 2.5, the peptide forms higher-order aggregates that are likely tetramers. One potential explanation for the difference between these findings and earlier data reported by Hodges (Lau *et al.* 1984; Zhu *et al.* 1993) might be in the values used for the partial specific volume and density. Using the H_2O–D_2O difference method (Edelstein & Schachman 1973), we determined the partial specific

Table 1. *Equilibrium sedimentation of coil-Xaa peptides*

(Conditions: 10 mM MOPS, pH 7.5. The molecular masses were determined using a partial specific volume calculated by the residue weighted average method (Cohn & Edsall 1943; Harding *et al.* 1992) as well as the H_2O–D_2O difference method (Edelstein & Schachman 1973) and found to agree within 0.01 ml g^{-1}. The partial specific volume for each of these peptides is 0.76 ml g^{-1}, except as noted. Solution densities were estimated by solute concentration-dependent density tables in the CRC Handbook of Chemistry and Physics. We estimate that the uncertainty in these measurements arises chiefly from the uncertainty in the partial specific volume, leading to an uncertainty of 10–20% in the observed molecular masses.)

peptide	monomeric MM	observed MM
coil-Ala	3331	10500
coil-Arg	3416	10800
coil-Trp	3446	10800
coil-Ser[a]	3347	10258
coil-V_aL_d	3218	10200
coil-L_aL_d	3274	9700
Asn_{16}-coil-v_aL_d	3233	7100[b]

[a] Data are from Lovejoy *et al.* (1993).

[b] Mean value of runs at rotor speeds of 20, 30, and 40 krpm. The partial specific volume was calculated to be 0.75 ml g^{-1}.

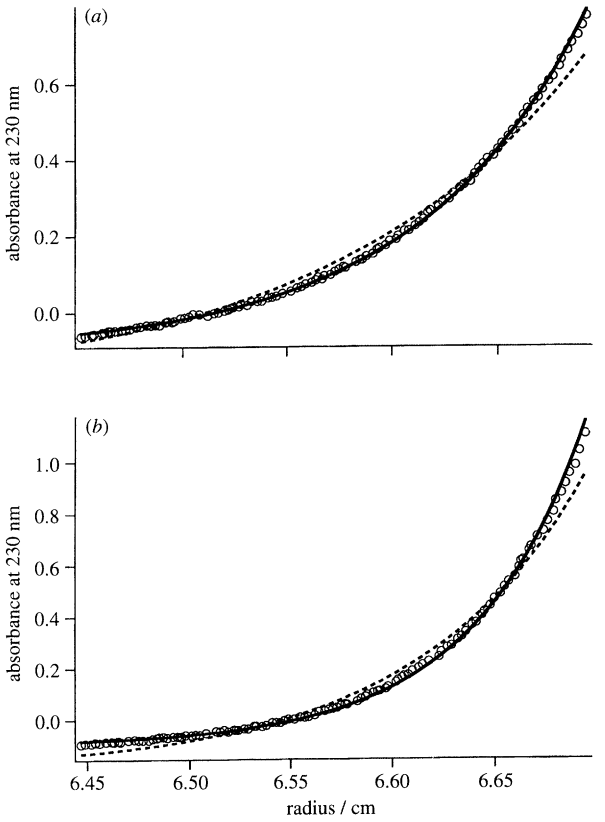


Figure 3. Sedimentation equilibrium of the TM-43 peptide. Circles represent the raw data, a solid line shows the theoretical fit to trimer, and a dashed line shows the theoretical fit to dimer. The data in the top panel were acquired at 35000 rpm. The data in the bottom panel were acquired at 48000 rpm. The conditions were 60 μM TM-43, 1.1 mKCl, pH 7.0.

Table 2. *Equilibrium sedimentation of TM-43 peptide*

(Samples were run at initial loading concentrations of 60 μM and 600 μM in 50 mM potassium phosphate, except as noted. The molecular mass of the monomer is 4632.The prevalent species was determined by monomer-*n*mer equilibria. Molecular mass calculated assuming only one species in solution. The partial specific volume was calculated by the H_2O–D_2O difference method (Edelstein & Schachman 1973) to be 0.76 ml g^{-1} in 50 mM potassium phosphate, 1.1 M KCl, and 0.75 ml g^{-1} in 10 mM MOPS.)

pH	[KCl] / M	prevalent species	single-species molecular mass	association state
7.0	1.1	trimer	13700	3.0
7.0	0.1	trimer	14200	3.1
2.5	1.1	tetramer	19100	4.1
2.5	0.1	tetramer	18000	3.9
7.5[a]	0.0	trimer	13400	2.9
7.5[a]	0.1	trimer	14600	3.1

[a] 10 mM MOPS buffer.

volume of TM-43 to be 0.76 ml g^{-1} under the conditions used here. The value used by Lau *et al.* (1984) was estimated to be 0.70 ml g^{-1}; the employment of this low value will lead to an underestimation of the aggregate's molecular mass. Solution densities used by Hodges and co-workers (Lau *et al.* 1984; Zhu *et al.* 1993) were not reported.

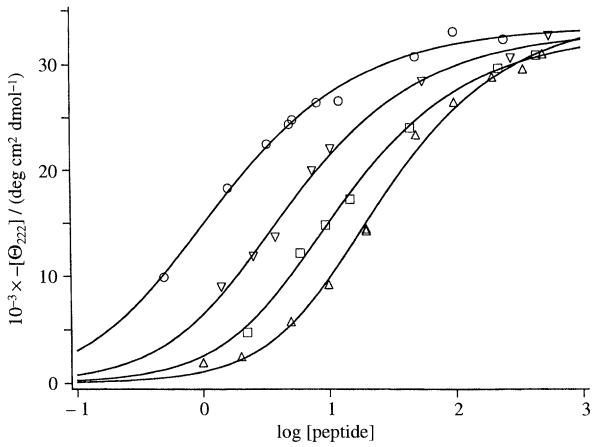


Figure 4. The dependence of mean residue ellipticity, $[\theta_{222}]$, on concentration for coiled-coil peptides. The raw data are shown as symbols: (circles) coil-Ala, (inverted triangles) coil-Ser, (squares) coil-Asp, (triangles) coil-D-Ala. The calculated fits are shown as solid lines. The Hill coefficients are presented in table 3.

(*b*) *Concentration dependence of the* CD *spectra of the original coiled-coil peptides*

In our earlier studies, self association of the peptides was measured in the presence of 5.0 M urea, where the oligomeric forms of these peptides are considerably less stable. Under these conditions, the concentration-dependent equilibrium between fully non-helical monomers and helical oligomers can be monitored readily by measuring the concentration dependence of their CD spectra. Figure 4 illustrates the concentration dependence of the helical content of several coiled-coil peptides as monitored by their mean residue ellipticity at 222 nm $[\theta_{222}]$. These curves can be analysed by a monomer–dimer–trimer equilibrium :

$$\begin{matrix} & K_2 & & K_1 & \\ \mathrm{T} & \rightleftharpoons & \mathrm{D} + \mathrm{M} & \rightleftharpoons & 3\mathrm{M}, \end{matrix}$$

$$K_1 = [\mathrm{M}]^2/[\mathrm{D}];\ K_2 = [\mathrm{D}]\,[\mathrm{M}]/[\mathrm{T}]. \qquad (7)$$

Two posible factors limit this equilibrium scheme: if K_1 is much smaller than K_2, then monomers and dimers would be the only significantly populated species in the transition zone and trimers would be formed only at considerably higher concentration. In this case, the Hill coefficient for the process would be 2.0, and the curves would be indistinguishable from simple monomer–dimer equilibria. Conversely, if K_2 is much smaller than K_1, then the concentration of dimers would be very small (relative to monomers and trimers) in the transition zone. In this case the Hill coefficient would be 3.0 and the data could be analysed as a cooperative monomer–trimer equilibrium. If the Hill coefficient for the process falls between these two limiting values, then K_1 and K_2 are reasonably close in magnitude and can be determined through careful analysis of the curve.

The Hill coefficients (n) for the 20 previously

Table 3. *Helix formation parameters and Hill coefficients for coiled-coil peptides*

(The values of $\Delta\Delta G_\alpha$ and n were determined using previously published data (O'Neil & DeGrado 1990; Fairman *et al.* 1992) and for the charged residues, have been corrected for electrostatic effects by extrapolating the value of $\Delta\Delta G_\alpha$ to 1.0 M NaCl.)

amino acid	Hill Coefficient	$\Delta\Delta G_\alpha$ (old) kcal mol^{-1}	$\Delta\Delta G_\alpha$ (new kcal mol^{-1}
Ala	2.25 (0.15)	−0.77	−0.71
D-Ala	2.42 (0.21)	0.18	0.34
Arg	2.34 (0.34)	−0.68	−0.70
Asp	2.37 (0.15)	−0.15	−0.10
Asn	2.19 (0.09)	−0.07	−0.01
Cys	2.51 (0.35)	−0.23	−0.22
Gln	2.99 (0.42)	−0.33	−0.33
Glu	2.40 (0.14)	−0.27	−0.21
Gly	2.90 (0.26)	0.00	0.00
His	2.22 (0.12)	−0.06	0.03
Ile	2.36 (0.17)	−0.23	−0.17
Leu	3.15 (0.63)	−0.62	−0.52
Lys	2.20 (0.26)	−0.65	−0.58
Met	2.46 (0.27)	−0.50	−0.42
Phe	1.90 (0.18)	−0.41	−0.37
Ser	2.31 (0.22)	−0.35	−0.27
Thr	2.23 (0.21)	−0.11	−0.09
Tyr	2.82 (0.15)	−0.17	−0.06
Trp	1.93 (0.68)	−0.45	−0.45
Val	2.55 (0.21)	−0.14	−0.16

reported coiled-coil peptides were each determined as described in the Methods (see table 3) from our published data and found to be 2.41 ± 0.07 (standard error of the mean). Two peptides were found to have Hill coefficients as low as 1.9 (coil-Phe and coil-Trp) and four had Hill coefficients of 2.8–3.15 (coil-Tyr, coil-Leu, coil-Gly and coil-Gln). However, the values of n for these peptides had larger relative uncertainties (see table 3), suggesting that deviations from the mean arose from errors in the data sets for these peptides rather than actual differences in the cooperativity. To test this idea further, we selected one peptide – coil-Tyr – for further study. A triplicate data set gave a Hill coefficient of 2.3 ± 0.1, suggesting that the outliers represented error in our previous data rather than actual differences in the cooperativity of association. This is structurally reasonable because the helix–helix interface should be very similar for each of the peptides as the changes in their sequences occur on their solvent-exposed faces.

A Hill coefficient of 2.4 indicates that the values of K_1 and K_2 are of similar magnitude. This was confirmed by fitting the individual data sets for the peptides to a monomer–dimer–trimer equilibrium as described in Methods. The average value of K_2/K_1 for the entire set of peptides was 0.6 corresponding to an energetic difference of 0.3 ± 0.15 kcal mol^{-1} (standard error of the mean).

To obtain estimates of the helix propensities of the amino acids from the monomer–dimer–trimer equilibria it is useful to consider how a change in the guest residue would affect the equilibrium constants, K_1 and K_2. Consider a given peptide with a given K_1 and K_2. Now another peptide with a different guest residue will have a monomer–dimer equilibrium constant, K_1, which is related to K_1 for the original peptide by equation (8):

$$K_1' = K_1 \, e^{-(2\Delta\Delta G\alpha/RT)}, \quad (8)$$

in which $\Delta\Delta G_\alpha$ is the difference in the free energy of dissociation between the two peptides (on a 'per monomer' basis), and the factor of two is included because two monomers join to form a dimer. After our earlier treatment we associate this free energy difference ($\Delta\Delta G_\alpha$) primarily with the difference in the propensity of helix formation for the two amino acids because the mutation occurs on the solvent-exposed face of the helix at a site distant from the helix–helix interface. Further, the dissociation constant for adding a single random-coil monomer to the helical dimer in the second step is given in equation (9) by analogy to equation (8)

$$K_2' = K_2 \, e^{-(\Delta\Delta G\alpha/RT)}. \quad (9)$$

However, in this step only one peptide switches from a random-coil monomer to a helical dimer so the value of $\Delta\Delta G_\alpha$ is not multiplied by two. We assume that $\Delta\Delta G_\alpha$ is identical for each monomer in the trimer because the local environment of the host sites are extremely similar as judged by the crystal structure. Using this treatment we obtained $\Delta\Delta G_\alpha$ for each of the amino acids referenced to Gly.

Table 3 provides the values of $\Delta\Delta G_\alpha$ for the commonly occurring amino acids derived from the simple monomer–dimer equilibria versus the more complete monomer–dimer–trimer equilibria (a plot is displayed in figure 5). The data are linearly related by the equation [$\Delta\Delta G_\alpha$ (new) $= 1.006 \times \Delta\Delta G_a$ (old) $- 0.05$; $r^2 = 0.98$]. Within our experimental error (0.1 kcal mol^{-1}), there are no significant differences between the two data sets.

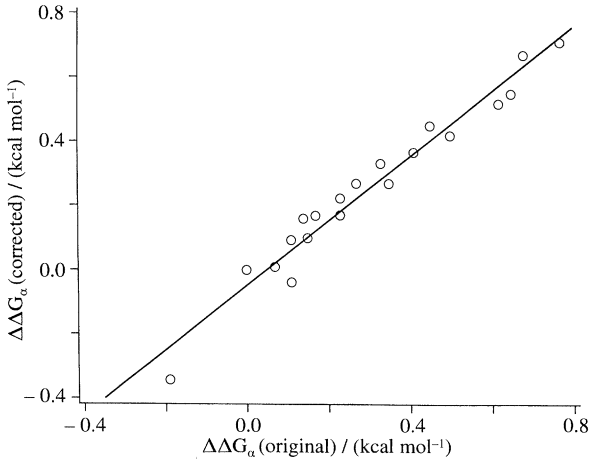


Figure 5. Comparison of revised and original values of $\Delta\Delta G_\alpha$. The individual data points are shown as circles, the linear regression is shown as a solid line.

4. DISCUSSION

The data in this paper clearly demonstrate that the peptides in this series, which contain Leu at positions 'a' and 'd', Glu at position 'e' and Lys at position 'g' of the coiled coil, exist in a non-cooperative monomer–dimer–trimer equilibrium. The relative amounts of the monomeric, dimeric and trimeric species as a function of the total peptide concentration for coil-Ala are shown in figure 6. We also explored the features that cause the peptides to form trimers rather than the originally designed dimers. Replacing Leu with Val at each 'a' position led to peptides that still formed trimers, and preliminary measurements of the concentration dependence of this peptide's CD signal indicate that the association is more cooperative; the addition of a monomer to a preformed dimer to form a trimer is more favourable than the initial dimerization of the two monomers. In contrast, addition of a single Asn residue at an 'a' position caused the peptide to form only dimers. This result parallels results previously described by Kim, Alber and their coworkers for the coiled-coil of GCN4 (O'Shea *et al.* 1991, 1993). The structural basis for this result can be appreciated by considering the effect of burying an Asn at the helix–helix interface of a dimer versus a trimer. In a dimer, the carboxamide side-chain from neighboring Asn residues can form a hydrogen bond between the carbonyl of one residue and the $-NH_2$ of the other (O'Shea *et al.* 1991). More importantly, having made these stabilizing interactions, the remaining portions of this polar side-chain can be hydrated through interactions with water. Model building suggests that in a parallel trimer, the same side chain–side chain hydrogen bonding can be realized. However, the Asn is fully buried in the interior of the structure without forming hydrogen bonds to all its polar functions. If the peptide forms an antiparallel trimer, as in coil-Ser, two Asn residues originating from the parallel pair of helices can form stabilizing H-bonded interactions, but the third Asn would be forced to be buried among Leu residues. Therefore the dimer is stabilized over the trimer by hydration effects when a single polar residue is introduced into the sequence. Thus, this change provides specificity to the structure, albeit at the price of thermodynamic stability. (Our preliminary data indicate that the addition of Asn destabilizes the free energy of dimerization by several kcal mol^{-1}.)

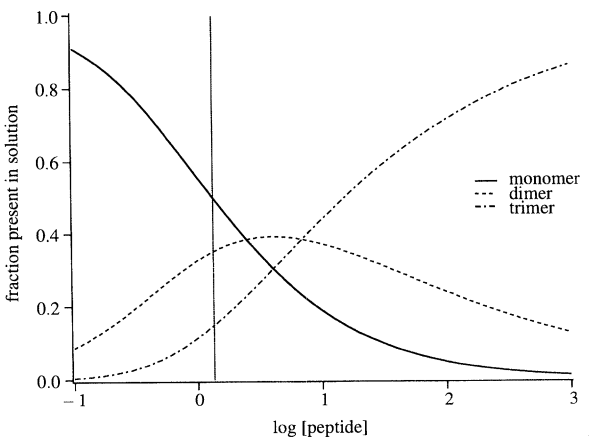


Figure 6. Oligomer population versus peptide concentration (μM) for coil-Ala. The curves are calculated from the equilibrium constants of the concentration dependent CD data. A solid line represents monomer, a dashed line represents dimer, and a dot-dashed line represents trimer. The vertical line represents the midpoint concentration of the association curve of coil-Ala.

From the perspective of *de novo* protein design, the non-specificity of the aggregation of coil-Ser was far from optimal. In a designed protein one seeks to obtain a structure that has a unique conformational state or association state; unlike the non-cooperative assembly seen for these peptides. If one wishes to stabilize a parallel dimer relative to a trimer, the addition of an Asn residue at an 'a' position appears to be a successful strategy. Alternatively, the addition of a Cys residue to form a disulphide-bonded dimer has been reported by Hodges to stabilize the dimer, although some higher-order oligomers were also formed (Zhou *et al.* 1993). The addition of β-branched amino acids, however, may stabilize the trimeric state although we have not yet investigated this in detail. Perhaps the most straightforward method to stabilize an antiparallel trimer is to entropically stabilize the three-helix bundle state through the incorporation of links between the helices. Work along these lines is showing considerable progress in our labs.

The primary motivation of our original design of two-stranded coiled coils was to design a system for exploring the helix propensities of the 20 commonly occurring amino acids. The finding that the peptides we had designed formed trimers as well as dimers, necessitated a thorough re-examination of our data. This analysis shows that the previously reported values of $\Delta\Delta G_\alpha$ obtained using a simple monomer-dimer treatment were, allowing for experimental error, indistinguishable from the values obtained in this work using a monomer–dimer–trimer treatment. This is because the values of $\Delta\Delta G_\alpha$ are obtained at the midpoint of the transition, where the amount of peptide in the trimeric state is about 15 % of the total (see figure 6). However, explicit treatment of the effect of trimer formation does remove a small (~ 0.05 kcal mol^{-1}), positive systematic error in the $\Delta\Delta G_\alpha$ values. Also, it is worth noting that although the new $\Delta\Delta G_\alpha$ values result in a different rank ordering of the helix propensities of the various amino acids, the absolute differences responsible for these rank changes are well within experimental uncertainty and, in fact, the correlation with other scales for helix formation (Park *et al.* 1993; Chakrabartty *et al.* 1994; Munoz & Serrano 1994) remains excellent and should continue to be a valuable aid in protein design.

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