ABSTRACT: The study and successful design of coiled-coil protein structural motifs have provided much insight into the rules governing protein folding and stability. In this work we use a thermodynamic approach to quantitate the rules that govern the specific oligomerization of coiled coils. We have designed a highly stable trimeric coiled coil by placing valine residues at each a position and leucine residues at each d position of the heptad repeating unit. The peptide forms a very stable trimer as determined by sedimentation equilibrium, and the concentration dependence of its circular dichroism spectrum follows a cooperative monomer/dimer/trimer equilibrium with the dimer state as a highly unstable intermediate. Its guanidinium chloride denaturation curve was collected at several peptide concentrations, and analysis of the data confirms the cooperativity of the trimerization process and provides a free energy of stabilization of $-18.4$ kcal mol$^{-1}$ for the trimer. The heat capacity, $\Delta C_p$, was measured by global analysis of thermal unfolding data collected at a number of guanidinium chloride concentrations. Guanidinium chloride induces cold denaturation in the thermal unfolding curves, providing a reasonably well-determined value for $\Delta C_p$ of 750 cal deg$^{-1}$ mol$^{-1}$. This translates to a $\Delta C_p$ of 8.6 cal deg$^{-1}$ mol$^{-1}$ per residue and corresponds well to that expected of a coiled coil with a well-defined tertiary structure.

The $\alpha$-helical coiled coil provides an excellent model system for investigating the forces that shape the structure of proteins. This structural motif has a geometric seven-residue repeat, which is generally reflected in the hydrophobicity of its amino acid sequence. A number of years ago, Hodges and coworkers prepared a series of polyheptapeptides with the sequence (Hb-Hp-Hp-Hb-Hp-Hp-Hp)$_n$, in which Hb and Hp are hydrophobic and hydrophilic amino acids and the subscripts refer to the position in the heptad repeat (Lau et al., 1984). These peptides self-assembled into highly helical oligomers, showing the importance of hydrophobic interactions in the assembly process (Figure 1a). More recently, the nature of the apolar amino acid residues at the a and d positions has been shown to have an important influence on the oligomerization state of GCN4-based coiled coils (Harbury et al., 1993). As the peptides associate, they seek to fully bury their hydrophobic residues while simultaneously adopting low-energy rotamers and optimizing their van der Waals interactions. The observed structures clearly reflect a compromise between optimizing these individual forces, as the crystal structure of a tetrameric coiled coil formed by a peptide with Leu at a and Ile at d contains a hole running down the central axis of the structure (Harbury et al., 1993). Breaks in the hydrophobic period can also have an important influence on the oligomerization state of the structures because it provided better dehydration of the hydrophilic side chains. As in the anticipated dimer, the trimer appears to be favored over dimeric interactions only if the structure is parallel in nature. Based on the early literature, it was assumed that these peptides would form parallel dimers, but a crystal structure of one member of this series of peptides, coil-Ser, showed an antiparallel trimeric structure (Lovejoy et al., 1993). The trimer appears to be favored over dimeric structures because it provided better dehydration of the hydrophobic side chains. As in the anticipated dimer, the trimer contains two helices docked in a parallel orientation with their apolar Leu residues associated in a hydrophobic interaction and salt bridges between Glu and Lys residues and hence does not require complete burial of the hydrophilic group (O’Shea et al., 1991, 1993; Betz et al., 1995).

Our own work has focused on a series of coiled coils modeled after one of Hodges’ original design (O’Neil & DeGrado, 1990; Betz et al., 1995). It contains Leu residues at each a and d position and Glu and Lys at e and g, respectively. These charged residues can undergo favorable interhelical electrostatic interactions only if the structure is parallel in nature. Based on the early literature, it was assumed that these peptides would form parallel dimers, but a crystal structure of one member of this series of peptides, coil-Ser, showed an antiparallel trimeric structure (Lovejoy et al., 1993). The trimer appears to be favored over dimeric structures because it provided better dehydration of the hydrophobic side chains. As in the anticipated dimer, the trimer contains two helices docked in a parallel orientation with their apolar Leu residues associated in a hydrophobic interaction and salt bridges between Glu and Lys residues.
at the helix–helix interface. However, a third helix docks in an antiparallel orientation against the parallel pair. Its Leu residues associate in layers with those from the other two helices forming an extensive hydrophobic core. The antiparallel orientation was unexpected because in this geometry the electrostatic interactions between this helix and its neighbors are potentially destabilizing and consist of one helix–helix interface that is rich in Lys residues, while the other interface is rich in Glu residues. While such an arrangement should be destabilizing at neutral pH, the peptide was crystallized at pH 5, near the pKₐ of the Glu residues. At this pH some of the Glu residues are protonated and might form stabilizing hydrogen bonds with deprotonated Glu residues. In fact, two such hydrogen-bonded pairs, between residue Glu-A27 and Glu-C6, and Glu-A24 and Glu-A20 (numbering from the Protein Data Bank, entry 1COS), are apparent in the crystal structure based on the distance of their carboxylate oxygen atoms. Consistent with this interpretation, fluorescent derivatives of coil-Ser adopt parallel rather than antiparallel trimers in aqueous solution at neutral pH (Wendt et al., 1995).

A detailed study of the association of coil-Ser in aqueous solution showed that it exists in a noncooperative monomer/dimer/trimer equilibrium (Hill coefficient = 2.3), in which ΔG° for the formation of a dimer from two monomers was approximately equal to ΔG° for the formation of the trimer from a monomer and a dimer (Lovejoy et al., 1993). We therefore were interested in determining what changes could be made to the sequence of coil-Ser to provide it with a stronger preference for either a dimeric or trimeric oligomerization state. A single Asn or Ala residue at an a position is capable of favoring the dimeric oligomerization state (Betz et al., 1995), but less has been known concerning the features that might uniquely stabilize the trimeric state. In this paper, we demonstrate that a derivative of coil-Ser with Val at each a position (coil-VₐLₐₐ) (Figure 1) cooperatively assembles into trimers.

EXPERIMENTAL PROCEDURES

Modeling. The coil-VₐLₐₐ peptide was modeled as two- and three-helix coiled coils using the programs InsightII and Discover (Biosym). The method for generating coiled-coil structures from first principles has been described previously (Dieckmann, 1995; Betz & DeGrado, 1996). A dimeric model was also created beginning with the coordinates from the crystal structure of GCN4 (O'Shea et al., 1991). The model was also created beginning with the coordinates from (Dieckmann, 1995; Betz & DeGrado, 1996). A dimeric and three-helix coiled coils using the programs InsightII and Discover (Biosym). The method for generating coiled-coil structures from first principles has been described previously (Dieckmann, 1995; Betz & DeGrado, 1996). A dimeric model was also created beginning with the coordinates from the crystal structure of GCN4 (O'Shea et al., 1991). The side chains were replaced with those of coil-VₐLₐₐ utilizing the same side chain rotamers as found in the GCN4 structure.

Peptide Preparation. The coil-VₐLₐₐ peptide was prepared using Fmoc-protected amino acids and techniques described previously (Choma et al., 1994). Peptide concentration was determined either by amino acid analysis (Liu & Boykins, 1989) or ninhydrin analysis (Rosen, 1957).

Sedimentation Equilibrium Ultracentrifugation. Experiments were performed using an An-60 Ti rotor in a Beckman Model XLA ultracentrifuge at 25 °C. The peptide loading concentration was 60 μM in 10 mM MOPS, 1 pH 7.5, and 150 mM NaCl. The NaCl is added to avoid charge density artifacts, and its addition has no appreciable effects on the stability or oligomeric state of the peptide. Data for the peptide were collected using six-channel Epon, charcoal-filled centerpieces with a 12 mm path length containing 110 μL samples and 125 μL buffer references. The samples were centrifuged at 25 000, 35 000, and 45 000 rpm, and the protein distribution was monitored at a wavelength of 242 nm. Ten successive radial scans were averaged using a 0.001 cm step size, and equilibrium was assumed if no change in distribution was observed over intervals of 2 h.

Data analysis software running under Igor (Wavemetrics, Lake Oswego, OR) and using the algorithm of Michael L. Johnson (Johnson et al., 1981) was a gift from Preston Hensley (Smith Kline Beecham, King of Prussia, PA). Data analysis was also done using the HID program from the Analytical Ultracentrifugation Facility at the University of Connecticut. The partial specific volume was calculated from the weight average of the partial specific volumes of the individual amino acids (Cohn & Edsall, 1943).

Circular Dichroism. Data were collected using an Avi 62DS circular dichroism spectropolarimeter equipped with a thermoelectric device for temperature control. All measurements were made in 10 mM MOPS, pH 7.5, at 25 °C. Data for the GuHCl denaturation experiments were collected after sufficient time was allowed for the samples to reach equilibrium. Data for the thermal melts were collected at 222 nm using a 1° step, a bandwidth of 1.5 nm, an equilibration time of 2.5 min, and a data averaging time of 1 min. Thermal unfolding experiments were tested for thermodynamic equilibrium by requiring exact overlapping unfolding curves as a function of equilibration time.

Peptide concentration dependence data and GuHCl denaturation data were fit by nonlinear least-squares methods to various equilibrium schemes using a PC version of the program MLAB (Civilised Software, Bethesda, MD) (Knott, 1979). The formalism used in the curve fitting has been described previously (Fairman et al., 1995). Global fits using the thermal unfolding data were carried out as described below.

The following relationship describes a monomer/trimer equilibrium scheme:

\[ \theta_{obs} = \theta_{mon} \frac{[\text{monomer}]}{[P_{tot}]} + \theta_{tr} \frac{3[\text{monomer}]^3}{K_3[P_{tot}]} \]  

The variation of [θ]obs on GuHCl concentration at constant temperature was treated using the following linear function (Santoro & Bolen, 1988) as described previously (Fairman et al., 1995) for a monomer/n-mer equilibrium:

\[ \theta_{obs} = \theta_{mon} \frac{[\text{monomer}]}{[P_{tot}]} + \theta_n \left( \frac{n[\text{monomer}]^n}{\exp \left( \frac{\Delta G_{H_2O}^{\Pi} + m[\text{GuHCl}]}{-RT} \right)} \right) \]  

where \( \Delta G_{H_2O}^{\Pi} \) is the free energy of stability in the absence of GuHCl, \( m \) is the dependence of stability of GuHCl concentration (in units of kcal mol\(^{-1}\) M\(^{-1}\)), \( T \) is the temperature (in kelvin), \( n \) is the oligomerization state, and \( R \) is the universal gas constant. For experiments at variable temperature the term \( \Delta G_{H_2O}^{\Pi} \) is replaced by the Gibbs–Helmholtz function:

1 Abbreviations: CD, circular dichroism; GuHCl, guanidinium chloride; SE, sedimentation equilibrium; MOPS, 3-morpholinopropanesulfonic acid.
RESULTS AND DISCUSSION

Modeling. coil-VAld was modeled as two- and three-stranded coiled coils to help to predict the structural features that might differentially stabilize these two association states. Because we were concerned with the association of the peptide at neutral pH, only parallel orientations were considered. At this pH, the Glu and Lys residues should be fully ionized, and repulsive electrostatic interactions should destabilize alternate antiparallel arrangements. The structures of the dimer and trimer were assembled from first principles using methods for the construction of coiled coils described previously (Dieckmann, 1995; Betz & DeGrado, 1996). This method requires the choice of a fixed rotamer for each interior residue. Val is known to adopt a single rotamer in α-helices (χ1 = 165°) (McGregor et al., 1987), and this value was used in all models (where χ1 for Val is defined by atoms N, Cα, Cβ, and Cγ). Leu can adopt two low-energy conformations in α-helices (McGregor et al., 1987), so two different models were generated for each oligomerization state: one in which all Leu residues have χ1 = −175° and χ2 = 75° (dimer) and a second model with χ1 = −60° and χ2 = −175° (dimer+) (for Leu, χ1 is defined by N, Cα, Cβ, and Cγ and χ2 by Cα, Cβ, Cγ, and Cδ). To test the appropriateness of this modeling procedure, we also constructed a model of the dimer utilizing the backbone coordinates from the crystal structure of GCN4. The rmsd’s between this model and the two dimeric coiled-coil models generated from first principles (Table 1) indicate that our modeling procedure generates structures with overall geometries that agree well with those observed in naturally occurring proteins.

As shown in Figure 2, efficient packing of the hydrophobic residues was observed in both the dimeric and trimeric oligomerization states. However, calculations based on the method of Eisenberg and McLachlan (Eisenberg & McLachlan, 1986; Eisenberg et al., 1989) suggest that hydration forces favor the formation of the trimer. The changes in solvation energy accompanying oligomerization are given in Table 1, computed for the two dimeric and trimeric models. On a per-monomer basis, the trimers consistently give more favorable solvation energies. In the models, efficient salt bridge is observed between Glu side chains at position e on one helix and Lys residues at position g of the preceding heptad on a neighboring helix in both the dimeric and trimeric structures.

Sedimentation Equilibrium. Sedimentation equilibrium ultracentrifugation indicates that coil-VAld forms very stable trimers in aqueous solution (pH 7.5, 10 mM MOPS, 150 mM NaCl, 25 °C) as reported previously (Betz et al., 1995) and as shown in Figure 3. The peptide sediments as a single homogeneous species at concentrations between approximately 10 and 100 µM, indicating that it does not significantly change its association state within this concentration regime. Analysis of the data indicates that it sediments with an apparent molecular weight of 10 076 ± 210. This value is within 4% of that calculated for the trimer.

GuHCl-Induced Unfolding Curves. The thermodynamic stability of coil-VAld was determined by monitoring its far-UV circular dichroism at 222 nm as a function of the concentration of GuHCl at a peptide concentration of 25 µM in 10 mM MOPS, pH 7.5 at 25 °C. The data are shown normalized to the fraction of trimer in Figure 4A. At low concentrations of GuHCl, the mean residue ellipticity at 222 nm ([θ]222) is approximately −36 000 deg cm²/dmol, consistent with a fully helical conformation. At high concentrations of denaturant [θ]222 approaches zero, indicating that the monomer is largely unstructured. The denaturation curves are all sigmoidal with a single inflection point, consistent with a two-state equilibrium in which intermediates are minimally populated at equilibrium.

An interpretation of the GuHCl unfolding curves for associating peptides requires a knowledge of the cooperat-
However, the value of $K_3$ of high degree of cooperativity of the curve and the covariance extremely well determined by the data, but because of the isotherm is optimally fit with a Hill coefficient of 3.0 (of the GuHCl-induced unfolding transition (Figure 5). The overall free energy of association, $RT \ln(K_2/K_3)$ is extremely well determined by the data, but because of the high degree of cooperativity of the curve and the covariance of $K_2$ and $K_3$, the values of the individual equilibrium constants are less well determined. The best fit to the data gave values of $K_2 = 5 \times 10^{-4}$ M and $K_3 = 2 \times 10^{-6}$ M. However, the value of $K_2$ can be varied 2–3-fold without significantly affecting the quality of the fit as long as $K_3$ was allowed to compensate in an opposite direction. On the basis of this observation, it appears that the ratio of $K_2$ to $K_3$ is at least 50, although it may be much larger. By contrast, the ratio of $K_2$ to $K_3$ for coil-Ser is near unity. Figure 5B shows that the data for coil-VaLd are not well fit when the ratio of $K_2$ to $K_3$ is forced to be 1.0 in the fitting algorithm.

Having established the cooperativity and stoichiometry of the association process, the entire GuHCl-induced denaturation curve (Figure 4) was evaluated to determine the free energy of trimerization of coil-VaLd in the absence of denaturant. This analysis involved making the usual assumptions that the transition was two state (in this case unfolded monomers going to α-helical trimers) and that the free energy of folding scaled linearly with [GuHCl] (Santoro & Bolten, 1988). The free energy of trimerization extrapolated to 0 M GuHCl was $-18.4 \pm 0.2$ kcal mol$^{-1}$. This free energy corresponds to a transition midpoint of 0.31 M for coil-VaLd. All three data sets were fit simultaneously to either monomer/trimer (--) or monomer/dimer (---) equilibrium schemes.

FIGURE 3: Sedimentation equilibrium analysis. Measurements were made using 60 µM coil-VaLd in 150 mM NaCl and 10 mM MOPS, pH 7.5, at 25 °C. Constants used for the data analysis are $r_{bar} = 0.761$ mL/g; and $\rho = 1.0017$ g/mL. The data are shown fit to a single ideal species.

FIGURE 4: GuHCl dependence. (A) Measurements were made using 25 µM coil-VaLd in 10 mM MOPS, pH 7.5, at 25 °C. The data are shown fit to monomer/trimer (--) and monomer/dimer (---) models using a linear function for [GuHCl] dependence and a linear function for the unfolded baseline. The curve-fitting parameters and their associated errors for the monomer/trimer model are as follows: $K_3 = 3.2 \pm 1.4 \times 10^{-14}$ M$^2$ [$\Delta G_{H_2O} = -18 400$ cal mol$^{-1}$; $\Delta G_2 = -12 900$ cal mol$^{-1}$]; $m_{[GuHCl]} = -2740 \pm 160$ cal mol$^{-1}$ M$^{-1}$; $\theta_{max} = 36 600 \pm 500$ deg cm$^2$ dmol$^{-1}$; $\theta_{min} = -2690 \pm 840$ deg cm$^2$ dmol$^{-1}$; monomer slope $= -420 \pm 160$ deg cm$^2$ dmol$^{-1}$ M$^{-1}$. (B) Measurements were made using 5, 25, and 100 µM coil-VaLd. All three data sets were fit simultaneously to either monomer/trimer (--) or monomer/dimer (---) equilibrium schemes.
The quality of the fits becomes sensitive to the association scheme if the GuHCl-induced unfolding curves are collected at several different peptide concentrations (Figure 4) and fit globally using the method described in Experimental Procedures. Clearly, a monomer/trimer scheme provides a better fit than that for a monomer/dimer scheme. If the degree of oligomerization is treated as an adjustable parameter in the global fit, a value of 3.18 ± 0.25 is obtained for the monomer/station.

Thermal Unfolding. The thermal denaturation of proteins provides information concerning the nature and cooperativity of the interactions stabilizing their structures. Molten globule proteins tend to have rather diffuse thermal unfolding curves, with low values of $\Delta C_p$ and $\Delta H^\circ$, while native proteins have cooperative unfolding transitions with a larger $\Delta C_p$ and $\Delta H^\circ$. coil-V a L d shows a cooperative thermal unfolding ($\Delta H^\circ = -39.2 \pm 0.2 \text{ kcal mol}^{-1}$ at 25 °C) transition in the absence of GuHCl. To determine $\Delta C_p$ for the transition, the unfolding curves were repeated in the presence of increasing concentrations of GuHCl (Figure 6), which decreases the observed enthalpy of the transition but has been observed to have a rather minor effect on $\Delta C_p$ (Makhatadze & Privalov, 1992). The apparent decrease in the plateau for the folded signal can be explained simply by the convergence of cold- and heat-denaturation thermal transitions and is an important effect for obtaining well-determined values for $\Delta C_p$ and $\Delta H$. The thermal unfolding curves were fit globally using the Gibbs–Helmholtz equation as described in Experimental Procedures. The free energy evaluated at 25 °C ($\Delta G = -18.8 \text{ kcal mol}^{-1}$) agrees well with that determined by the GuHCl denaturation method described above ($-18.4 \text{ kcal mol}^{-1}$). The computed value of $\Delta C_p$ for the transition is 750 cal deg$^{-1}$ mol$^{-1}$, and a sensitivity analysis indicated that this value was accurate to within approximately 100 cal deg$^{-1}$ mol$^{-1}$. Small, but nonrandom deviations in the quality of the fits probably reflect variations in $\Delta C_p$ with both temperature (Hagihara et al., 1994) and [GuHCl] (Makhatadze & Privalov, 1992). Expressed on a per residue basis, the change in the heat capacity difference between the folded and unfolded states amounts to 8.6 cal deg$^{-1}$ mol$^{-1}$ per residue.

Surprisingly, values of $\Delta C_p$ have been measured for only a few associating peptide systems, and these values vary greatly. Values of $\Delta C_p$ range from as high as 20.7 cal deg$^{-1}$ mol$^{-1}$ per residue for the VBP dimeric coiled coil measured by analysis of CD thermal unfolding curves (Krylov et al., 1994) to as low as 3.8 cal deg$^{-1}$ mol$^{-1}$ per residue for the GCN4 dimeric coiled coil (measured calorimetrically) (Thompson et al., 1993). Our work on the tetrameric coiled coil from Lac repressor, Lac21 (Fairman et al., 1995), gave an intermediate value of 14.8 cal deg$^{-1}$ mol$^{-1}$ per residue (Fairman, unpublished results). We have also measured a value of 8.8 cal deg$^{-1}$ mol$^{-1}$ per residue for a designed tetrameric coiled-coil peptide that shows native-like properties in solution (Betz & DeGrado, 1996). Probably the most careful thermodynamic analysis has been reported for melittin...
(Wilcox & Eisenberg, 1992; Hagihara et al., 1994). Wilcox and Eisenberg (1992) reported a range of values of $\Delta C_p$ of 9.3–13.2 cal deg$^{-1}$ mol$^{-1}$ per residue. This result was confirmed and more rigorously tested by Goto and colleagues (1994), who compared the values of $\Delta C_p$ obtained from van’t Hoff and calorimetric analyses. They found that $\Delta C_p$ for melittin has a significant temperature dependence, particularly at high temperatures, but otherwise behaved as a two-state system since the two methods gave good agreement.

CONCLUSIONS

The current challenge in protein design is choosing a primary sequence that will define a specific tertiary structure. The greatest progress in defining rules for tertiary structure has been in the design of coiled-coil protein folds, particularly in defining specific oligomerization states. We have been interested in gaining a thermodynamic understanding of these states. Toward this aim, we have designed a highly cooperative all-parallel trimeric coiled coil, coil-V$_{aLd}$, containing valine at $a$ positions and leucine at $d$ positions of each heptad repeat and have measured its thermodynamic parameters.

First, we show that the folding of coil-V$_{aLd}$ is highly cooperative as measured by following the acquisition of secondary structure as a function of peptide concentration. In contrast to GCN4-pVL (Harbury et al., 1993), a coiled coil also containing the motif V$_{aLd}$, and coil-Ser (Betz et al., 1995), the dimeric state is a highly unstable intermediate in the monomer/trimer equilibrium. The trimer state is very stable, exhibiting a free energy of $-18.4$ kcal mol$^{-1}$ as measured by global fits to [GuHCl] dependence of stability measured at several peptide concentrations.

To test the nature of the tertiary structure, we measured the thermal unfolding of the coil-V$_{aLd}$ peptide as a function of GuHCl concentration. Under these conditions we see both cold and heat denaturation as evidenced by the suppression of the signal at the low-temperature plateau in the data. These data were fit globally to the Gibbs–Helmholtz equation, assuming a two-state folding function, resulting in a $\Delta C_p$ of 8.6 cal deg$^{-1}$ mol$^{-1}$ per residue. This value is somewhat smaller than that observed for globular proteins with well-defined tertiary structures but well within the range of values of $\Delta C_p$ that have been observed for coiled coils. The wide range reported in the literature does not appear to correlate to any biophysical characteristic of this class of protein fold and merits further study.

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REFERENCES