Clustering of Large Hydrophobes in the Hydrophobic Core of Two-stranded α-Helical Coiled-Coils Controls Protein Folding and Stability*

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The de novo design and biophysical characterization of two 60-residue peptides that dimerize to fold as parallel coiled-coils with different hydrophobic core clustering is described. Our goal was to investigate whether designing coiled-coils with identical hydrophobicity but with different hydrophobic clustering of non-polar core residues (each contained 6 Leu, 3 Ile, and 7 Ala residues in the hydrophobic core) would affect helical content and protein stability. The disulfide-bridged P3 and P2 differed dramatically in α-helical structure in benign conditions. P3 with three hydrophobic clusters was 98% α-helical, whereas P2 was only 65% α-helical. The stability profiles of these two analogs were compared, and the enthalpy and heat capacity changes upon denaturation were determined by measuring the temperature dependence by circular dichroism spectroscopy and confirmed by differential scanning calorimetry. The results showed that P3 assembled into a stable α-helical two-stranded coiled-coil and exhibited a native protein-like cooperative two-state transition in thermal melting, chemical denaturation, and calorimetry experiments. Although both peptides have identical inherent hydrophobicity (the hydrophobic burial of identical non-polar residues in equivalent heptad coiled-coil positions), we found that the context dependence of an additional hydrophobic cluster dramatically increased stability of P3 (ΔT_m \approx 18 °C and Δ(urea)_{52} \approx 1.5 \text{ m}) as compared with P2. These results suggested that hydrophobic clustering significantly stabilized the coiled-coil structure and may explain how long fibrous proteins like tropomyosin maintain chain integrity while accommodating polar or charged residues in regions of the protein hydrophobic core.

Understanding protein folding remains a challenging problem: how does information encoded in the amino acid sequence translate into the three-dimensional structure necessary for protein function? Although hydrophobic interactions are generally accepted as the predominant source of free energy change that maintains the folded state, this non-specific stabilization does not describe how the “hydrophobic collapse” guides the formation of specific secondary structure (α-helices and β-sheets) in the final tertiary and quaternary structure in the native protein. The concomitant model suggests that the hydrophobic collapse restricts the conformation of the polypeptide chain into a “molten globule,” thus facilitating secondary structure folding in this limited conformational context. Examples of hydrophobic interactions participating in the early events of protein folding are observed via stopped flow fluorescence and nuclear magnetic resonance (NMR) studies in apomyoglobin (2) and cytochrome c (3), illustrating the importance of the packing of non-polar residues in stabilizing helix-helix interactions. Recently, non-polar residues have also been observed to form non-native hydrophobic clustering in denatured proteins (4, 5), and the authors postulated that non-native hydrophobic interactions can stabilize the long range order of the protein scaffold via an intermediate not observed in the folded state, thus indirectly guiding the extended polypeptide chain toward the correct native fold. Such an observation suggests that the amino acid sequence encodes for structural characteristics other than that of the native fold; in other words, the hydrophobic patterning in the sequence encodes the pathway that ultimately leads to the native functional state.

Considering that hydrophobic interactions mediate protein folding both in the folded and the unfolded state, several questions arise: 1) How does a cluster of non-polar residues contribute to stability? 2) Is the free energy derived from the burial of hydrophobic residues simply a sum of the energy derived from the removal of non-polar surface area from aqueous medium? 3) Does hydrophobic clustering enhance stability via favorable enthalpic, geometric packing, and van der Waals interactions?

The two-stranded α-helical coiled-coil is the simplest protein fold consisting of two amphipathic α-helices wound around one another forming a left-handed supercoil stabilized by hydrophobic burial (6, 7). All coiled-coils share a characteristic heptad (7-residue) repeat denoted as (abcdefg), in which non-polar residues occupy the a and d positions, forming an amphipathic surface where non-polar interactions allowed assembly of two-, three-, and higher oligomeric states (8). The quantitative contribution of 20 amino acids in positions a and d and their effects on protein stability and oligomerization state have been determined (9–11). In addition, the secondary structure formation and hydrophobic collapse of coiled-coils are tightly coupled and cooperative since single-stranded amphipathic α-helices are unstable in aqueous medium. This hydrophobic surface where amphipathic α-helices interact via hydrophobic interactions provides an ideal model to test the effects of hydrophobic clustering. We postulated that hydrophobic clustering in the core of coiled-coils would have a significant influence on secondary structure formation and protein stabil-
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EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification—Peptides were synthesized by automated solid-phase methodology described previously (12, 13) by conventional t-butylxycarbonyl chemistry (reviewed in Ref. 14). The peptides were synthesized on an Applied Biosystems model 430A peptide synthesizer as described previously (15). Briefly, the polypeptide chain was assembled on copoly(styrene, 1% divinylbenzene)-4-methyl-benzhydrylamine-HCl (MBHA) resin, 100-μm particle size, 300-Å inner diameter, 5-μmol peptide chain bound to resin) was then coupled onto the activated amino acid ester (4.0 equivalent excess as compared with 1.5 equivalent to mmol polypeptide chain bound to resin) for 5 min. The activated amino acid ester (4.0 equivalent excess as compared with mmol of polypeptide chain) and excess disisopropylglycylamine (1.5 equivalent to mmol polypeptide chain bound to resin) for 5 min. The activated amino acid ester, 5-μmol peptide chain bound to resin was then washed repeatedly with dichloromethane and dimethylformamide. Cleavage of the t-butylxycarbonyl and side chain-protecting groups and the subsequent release of the completed peptides from the MBHA resin support was achieved with hydrogen fluoride containing scavengers, 10% (v/v) anisole, and 1% (v/v) 1.2 ethanedithiol, magnetically stirred for 60 min in a reaction vessel with the temperature controlled at (−4 °C) by immersion in a sodium chloride water bath. The peptide resin was then washed three times with cold diethyl ether to remove scavengers and amino acid-protecting groups. Subsequent resin extraction with glacial acetic acid and overnight lyophilization yielded the crude peptide.

Crude peptides were purified by reversed-phase chromatography (reviewed in Ref. 16) on a Zorbax semipreparative SB-C8 column (250 × 9.4 mm inner diameter, 5-μm particle size, 300-A pore size) by linear AB gradient elution (0.2% increasing acetonitrile/min), where eluent A is 0.05% aqueous trifluoroacetic acid and eluent B is 0.05% trifluoroacetic acid in acetonitrile. The purification was carried out at room temperature with a constant flow rate of 2 ml/min. The purity and homogeneity of the peptide was verified by reversed analytical reversed-phase chromatography on a Zorbax analytical 300-A SB-C8 column (150 × 2.1 mm inner diameter, 5-μm particle size, 300-A pore size), by quantitative amino acid analysis (Beckman Model 6300 amino acid analyzer), and by electrospray mass spectroscopy on a Fisons Quattro (Fisons, Pointe-Claire, Quebec, Canada). Formation of the disulfide-bridged two-stranded homodimeric coiled-coil was obtained by overnight stirring in a 100 mM NH₄HCO₃ buffer, pH 8.5, and the desired product was purified by reversed-phase chromatography (described above).

Analytical Ultracentrifugation Equilibrium Experiments—Sedimentation equilibrium analysis was performed on a Beckman XLA analytical ultracentrifuge with absorbance optics at 274 nm for the detection of tyrosine. Samples were first dialyzed exhaustively against an aqueous solution of 100 mM KCl, 50 mM PO₄, pH 7.0 (benign buffer) at 4 °C. A 100-μl aliquot was loaded into the 12-mm Epon cell (charcoal-filled), and the initial loading concentrations of peptide stock solutions ranged from 50 to 500 μM in benign buffer. The samples were spun at 20 °C at 20,000, 25,000, and 35,000 rpm for 24 h to achieve equilibrium, as demonstrated by successive identical radial absorbance scans at 274 nm. The behavior of the peptide species at equilibrium is described by the following equation,

\[ M_{\text{obs}} = M_0 (1 - r^p) \]  

(Eq. 1)

where \( M_{\text{obs}} \) is the measured buoyant weight, \( M_0 \) is the molecular mass in daltons, \( r \) is the partial specific volume of the sample, and \( p \) is the density of the buffer solution. The partial specific volume of the sample and density of the buffer were calculated using SednTerm (version 1.06, University of New Hampshire) using the weighted average of the amino acid content. The peptide oligomerization behavior was determined by fitting the sedimentation equilibrium data from different initial loading concentrations and rotor speeds to various monomer-oligomer equilibrium schemes using WIN NonLIN (version 1.035, University of Connecticut), a non-linear least squares algorithm for equilibrium ultracentrifugation analyses (17).

Circular Dichroism Spectroscopy—Circular dichroism (CD) spectroscopy was performed on a Jasco-810 spectropolarimeter with constant N₂ flushing (Jasco Inc., Easton, MD). A Lauda circulating water bath was used to control the temperature of the optic cell chamber, where rectangular cells of 1-mm path length were used. The concentration of peptide stock solutions was determined by absorbance at 275 nm in 6 mM urea (extinction coefficient, \( \epsilon = 1420 \text{ cm}^{-1} \cdot \text{M}^{-1} \cdot \text{cm}^{-1} \), 1 tyrosine per peptide chain). For wavelength scan analysis, a 5 mg/ml stock solution of each peptide in 100 mM KCl, 50 mM PO₄, pH 7.0 (benign buffer) was diluted and scanned in the presence and absence of 50% trifluoroethanol (TFE).

Mean residue molar ellipticity was calculated using the equation,

\[ \theta_m = \frac{\theta_{222}}{100 \epsilon} \]  

(Eq. 2)

where \( \theta_m \) is the observed ellipticity in millidegrees, \( \theta_{222} \) is the mean residue molecular weight, I is the optical path length of the CD cell (cm), and \( \epsilon \) is the peptide extinction coefficient (mg/ml) per peptide chain. The calculated fraction of the unfolded state, \( U(t) \), and the temperature dependence of the mean residue ellipticity \( \theta_{222} \), was fitted to obtain fraction of the unfolded state, \( U(t) \), and post-translational (unfolded state, \( \theta_{222} \)) baseline corrections (19, 20),

\[ \theta_{222} = [1 - P(U)] \theta_{222}^{\text{eq}} + [P(U)] \theta_{222}^{\text{eq}} \]  

(Eq. 3)

where the pre- and post-translational baseline are assumed to be linearly dependent on temperature, and with \( \theta_{222}^{\text{eq}} \) and \( \theta_{222}^{\text{eq}} \) as 0 °C intercepts, respectively,

\[ \theta_{222}^{\text{eq}} = \theta_{222}^{\text{eq}} - m \cdot T \]  

(Eq. 4)

and

\[ \theta_{222}^{\text{eq}} = \theta_{222}^{\text{eq}} - m \cdot T \]  

(Eq. 5)

The calculated fraction of the unfolded state, \( P(U) \), is given by,

\[ P(U) = \exp(-\Delta G_{222}/RT) \]  

(Eq. 6)

where \( \Delta G_{222} \) is the apparent Gibbs free energy of folding described by the Gibbs-Helmholtz equation,

\[ \Delta G_{222} = \Delta H^{\text{obs}} - T \Delta S^{\text{obs}} - \Delta C_P (T - T_m) - T \ln(T/T_m^*) \]  

(Eq. 7)

where \( T_m \) is the temperature midpoint of the thermal transition, \( \Delta H^{\text{obs}} \) is the apparent enthalpy of unfolding, and \( \Delta C_P \) is the change in heat capacity associated with protein unfolding. Although \( \Delta C_P \) is temperature-dependent (21, 22), but in the narrow temperature range of our experiments (5–60 °C), this term is generally insensitive to changes (23). These thermodynamic parameters were fitted using the program Igor Pro (WaveMetrics, Inc.) with the protocol described in Ref. 20.

Chemical Denaturation Monitored by Circular Dichroism—For chemical denaturation experiments, the stock peptide solution was diluted with appropriate volumes of benign buffer and a stock solution of 10.0 mM urea in benign buffer to give a series of data points in increasing denaturant concentration. Data points were left to equili-

The abbreviations used are: CD, circular dichroism spectroscopy; DSC, differential scanning calorimetry; MBHA, copoly(styrene, 1% divinylnitrobenzene)-4-methylbenzhydrylamine-HCl; TFE, trifluoroethanol.
Peptide nomenclature, sequences, and schematics of the coiled-coil analogs used in this study. The top panel shows the sequence of two peptides, P3 and P2, which contain three and two clusters, respectively. The heptad repeat is denoted as *gabcdef*, which is shown above the first heptad of the coiled-coil. The bottom panel shows a schematic representation of the hydrophobic residues in positions *a* and *d*, where large hydrophobes are shown by *dark circles* (Leu, Ile), and small hydrophobes are shown by *open circles* (Ala). Three continuous large hydrophobic residues in the core positions *d, a, d* constitute a hydrophobic cluster (rectangular box).

### TABLE 1

Biophysical characterization of the oxidized and reduced hydrophobic cluster peptides

| Peptide Name | Hydrophobic Clusters | Schematic Representation of Hydrophobic residues at a and d positions |
|--------------|----------------------|---------------------------------------------------------------|
| P3           | 3 Clusters           | ![Schematic for P3]                                             |
| P2           | 2 Clusters           | ![Schematic for P2]                                             |

Peptides are named by the number of stabilizing hydrophobic clusters shown in Fig. 1.

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Differential Scanning Calorimetry—Excess heat versus temperature for the peptides was determined using a Microcal differential scanning calorimeter (Microcal, Northampton, MA). Sample concentrations ranged from 105 to 140 μM coiled-coil dimer, and peptides were dissolved in 10 mM KCl, 50 mM PO₄, pH 7.0, buffer. The sample solutions and buffer were filtered and degassed under vacuum and stored at 5 °C. Buffer scans were repeated until identical baselines were achieved. The heating rate was 60 °C/h, and the cooling rate was 90 °C/h with the excess heat monitored from 5 to 70 °C. Each sample was heated and cooled for three cycles to ensure folding reversibility. Data analyses were carried out in Microcal Origin software (Microcal DSC, version 1.2a) using a two-state model with change in heat capacity.

**RESULTS**

**Design of the α-Helical Coiled-coils with Different Hydrophobic Clustering**—The peptides used in this study were modeled on heptad sequences that had strong α-helical potential and a heptad repeat (*gabcdef*), where non-polar residues at positions *a* and *d* facilitate coiled-coil formation. In the design of these hydrophobic clustered peptides, we took advantage of the features of the successful α-helical coiled-coil models in our laboratory (6, 7, 9–11), for example, complementary packing in the protein core (26), balance of charged residues across the coiled-coil interface in heptad positions *e* and *g* (27, 28), and a flexible disulfide bridge linkage (29). The coiled-coil sequences consisted of 8 heptads (56 residues) based on 2 repeating heptad sequences: EXEAXK and KXEAXEG where positions *X* represent hydrophobic core positions occupied by Ala, Ile, or Leu in positions *a* or *d* (Fig. 1.). We defined a hydrophobic cluster as a consecutive string of three large non-polar residues.
(Ile or Leu) in the core positions of the coiled-coil. In our coiled-coils, non-polar residues Leu, Ile, Leu in the consecutive d, a, d heptad positions defined a stabilizing hydrophobic cluster. Our approach was to design two proteins with identical inherent hydrophobicity, i.e. identical number and character of non-polar residues in equivalent coiled-coil core positions but with a different arrangement, i.e. P3 having three hydrophobic clusters and P2 having two (Fig. 1, rectangular boxes). The N-terminal hydrophobic cluster of P3 was disrupted by an interchange of Ile at position 9 and Ala at position 16, both at heptad a positions, to give P2 (Fig. 1, bottom). Therefore, the two analogs have identical inherent hydrophobicity but different clustering patterns. The hypothesis is that the hydrophobic clusters are independent units that contribute to coiled-coil stability and folding when separated along the coiled-coil chain by consecutive strings of Ala residues (Fig. 1, open circles).

Thus, this pattern of large and small non-polar core residues helped distinguish the contribution of a hydrophobic cluster from inherent hydrophobicity. Interchain and intrachain ionic interactions were engineered by placing Lys and Glu at positions b, e, and g, resulting in ionic stabilization due to interchain electrostatic attractions (i to i’ + 5 or g to e’) and intrachain ionic attractions (i to i’ +3 or i to i + 4). To promote coiled-coil formation, a C-terminal disulfide bridge, Gly-Gly-Cys-Tyr linker was introduced to facilitate the formation of a parallel and in-register coiled-coil, and the single Tyr residue allows for protein concentration determination by UV spectrosopy. The disulfide bridge was distant from the N-terminal hydrophobic cluster under investigation (Fig. 1).

Secondary Structure Characterization by Circular Dichroism—Circular dichroism is a sensitive probe of secondary structural features, and this technique was used to detect the difference in helical content between the two peptides. Reduced P3 and P2 peptides were helical in benign condition (50 mM PO₄ (K₂HPO₄/KH₂PO₄), 100 mM KCl, pH 7.0, at 20 °C). The concentrations of P3 (○) and P2 (○) are 103 and 110 μM, respectively. deg, degree. B, sedimentation equilibrium of cluster peptides in benign condition at 20,000 rpm. The residuals are shown above the UV absorbance data, which were best-fitted to a single species (two-stranded disulfide-bridged coiled-coil model). Initial peptide concentrations for P3 and P2 were 100 μM.

FIG. 2. Biophysical characterization of the two-stranded disulfide-bridged coiled-coil peptides: secondary structural content and oligomeric state. A, circular dichroism spectra of cluster peptides in benign condition (50 mM PO₄ (K₂HPO₄/KH₂PO₄), 100 mM KCl, pH 7.0, at 20 °C). The concentrations of P3 (○) and P2 (○) are 103 and 110 μM, respectively. deg, degree. B, sedimentation equilibrium of cluster peptides in benign condition at 20,000 rpm. The residuals are shown above the UV absorbance data, which were best-fitted to a single species (two-stranded disulfide-bridged coiled-coil model). Initial peptide concentrations for P3 and P2 were 100 μM.

FIG. 3. Stability comparison of the P3 (three-cluster) and P2 (two-cluster) peptides. A, temperature melting of the coiled-coils monitored by circular dichroism spectroscopy at 222 nm. Experimental conditions were 50 mM PO₄ (K₂HPO₄/KH₂PO₄), 100 mM KCl, pH 7.0, buffer at a starting temperature of 5 °C. In B, urea denaturation experiments were carried out at 20 °C in a 50 mM PO₄ (K₂HPO₄/KH₂PO₄), 100 mM KCl, pH 7.0, buffer with increasing concentrations of urea as denaturant. Peptide P3 (○) and peptide P2 (○) had respective concentrations of 103 and 110 μM disulfide-bridged two-stranded-coiled. The ellipticity of peptide P3 was taken as fully folded to determine the fraction folded.
two-stranded coiled-coils P3 and P2 exhibited more helical structure, and the P3 coiled-coil with three hydrophobic clusters was fully folded (98% α-helical) at room temperature (Fig. 2 and Table I). The P2 coiled-coil with two hydrophobic clusters was only 65% folded at 20 °C in benign buffer, although more helicity was induced at 5 °C (Fig. 3A). P3 coiled-coil showed a [θ]222/208 ~ 1.02, indicative of a fully folded coiled-coil (32), whereas that of P2 coiled-coil is less than 1 (0.74) due to the presence of the single-stranded unfolded state (Fig. 2). Thus, without the presence of the third hydrophobic cluster, P2 did not fully fold in benign condition. In 50% TFE, both the disulfide-bridged peptides showed nearly 100% helical content, and the [θ]222/208 ratios for P3 and P2 were, respectively, 0.91 and 0.89, indicative of the single-stranded α-helical conformation (32). The significant helix induction for P2 in TFE, an increase of 35% helicity, showed the underlying high helical propensity of this sequence. However, P2 remained partially unfolded in benign condition because of insufficient hydrophobic stabilization in the hydrophobic core (having only two clusters).

**Sedimentation Equilibrium Analyses of Oligomeric States**—The packing of hydrophobic core residues had been shown to affect the oligomerization states of coiled-coils (9, 10). To determine that P2 and P3 had the same oligomerization state, sedimentation equilibrium analyses were carried out. Using different protein concentrations (from 50 to 500 μM) and rotor speeds (20,000–35,000 rpm), we found that the oligomerization behavior of coiled-coils P3 and P2 were best-fitted to that of a single homogeneous species with molecular weights consistent with a two-stranded coiled-coil (Fig. 2 and Table I). Overall, sedimentation equilibrium analyses showed that the interchange of hydrophobic residues Ile and Ala that distinguish P2 and P3 did not change the overall oligomerization state, i.e. these coiled-coils are two-stranded and do not assemble into high order oligomerization states. Therefore, we attributed the difference in helicity to a difference in stability due to the presence of an additional stabilizing hydrophobic cluster in peptide P3.

### Comparison of Coiled-coil Stability by Thermal and Urea Denaturation

The P3 coiled-coil with three hydrophobic clusters exhibited a highly co-operative unfolding similar to that of native proteins (Fig. 3). In contrast, P2 coiled-coil was only partially folded with marginal stability. We determined the stability of these two analogs using thermal and urea denaturation and found that P3 was significantly more stable than P2, with an increase of thermal midpoint of more than 18 °C and a corresponding increase in urea denaturation midpoint of ~1.5 M (Fig. 3 and Table II). To calculate the free energy difference between these two analogs, the linear extrapolation method was used to evaluate the chemical denaturation data (Table II) because it is difficult to estimate thermodynamics parameters ∆H and ∆Cp from non-ideal thermal melting curves (observed in P2). In addition, a more accurate protocol would be to estimate the free energy change from chemical denaturation because the slope around the urea denaturation transition is reliable (26). The free energy contribution of the N-terminal hydrophobic cluster in the P3 coiled-coil to stability was estimated to be 2.1 kcal·mol⁻¹ per coiled-coil, and this difference in stability can explain why P2 coiled-coil did not fully fold. The slope term m associated with the transition for P3 was significantly higher than that of P2 (Table II) and might describe the non-ideal behavior of the poorly formed secondary structure of P2. The m slope value generally correlates with the magnitude of changes in the non-polar accessible surface area between the

| Peptide name | t_m (melting) °C | [urea]1⁄2 M | m, slope | ∆G_H2O (CD) ΔH_DSC (CD) | ∆G_H2O (DSC) ΔH_DSC (DSC) |
|--------------|-----------------|-------------|----------|---------------------------|---------------------------|
| P3           | 41.6            | 2.10        | 1.35     | 2.92                      | 2.1                       |
| P2           | 23.1            | 0.65        | 0.67     | 0.86                      |                           |

Peptides are named by the number of stabilizing hydrophobic clusters shown in Fig. 1. t_m (CD) is the temperature at which there is a 50% decrease in molar ellipticity, [θ]222, compared to the fully-folded peptide with three clusters as determined by circular dichroism spectroscopy. [urea]1⁄2 is the denaturation midpoint of the two-state unfolding of an α-helical coiled-coil to a random coil, i.e. the urea concentration (M) required to achieve a 50% decrease in molar ellipticity, [θ]222, with a fully-folded coiled-coil taken as ~34,200 at 20°C.

m is the slope term defined by the equation ∆G = ∆G_H2O = −m [denaturant], and ∆G_H2O is the free energy of denaturation in the absence of denaturant, whereas ∆G is the free energy of unfolding at a given denaturant concentration.

∆G_H2O is the free energy difference contributed by a stabilizing cluster ∆G (CD) = ∆G (3 clusters − 2 clusters).

t_m (DSC) is the temperature midpoint associated with the transition peak during differential scanning calorimetry.

ΔH (CD) is the change in enthalpy derived from the thermal denaturation experiment using CD spectroscopy.

ΔH (DSC) is the change in enthalpy derived from the calorimetry experiment.

ΔC_p is the change in heat capacity associated with the denaturation transition observed in the differential scanning calorimetry experiment.

![Fig. 4. Stability profile of the three-clustered P3 peptide: thermal melt of P3 peptide as a function of temperature (●) overlaid on the differential scanning calorimetry profile (C). Experimental conditions were 50 mM PO₄ (KHOPO₄/KH₂PO₄), 100 mM KCl, pH 7.0, and peptide concentration was 105 μM disulfide-bridged two-stranded peptide.](image-url)
found that the hydrophobic destabilization of a Leu to Ala substitution is largely entropic at room temperature but becomes increasingly enthalpic at higher temperature.

Historically, hydrophobic cluster analysis structural prediction algorithm, based on the principles that hydrophobic amino acids cluster together in the native folded state, has been employed to good effect in identifying proteins with little sequence homology but with similar overall protein topology (reviewed in Ref. 38). Although hydrophobic cluster analysis does not predict the hydrophobic effect or protein stability, our results clearly show that significant stabilization can be achieved when hydrophobes cluster in the coiled-coil core. Supporting the results from these prediction programs, experimental data on GCN4 coiled-coil folding suggested that the high energy folding transition state is a hydrophobic collapsed form that contained little secondary structure (39), and therefore, the hydrophobic effect can be an early folding determinant of protein folding, whereas formation of helical secondary structure occurs later.

Hydrophobic clustering may play a significant role in the structure and function of long native coiled-coil proteins. In a recent study on the assembly of tropomyosin, a 284-residue coiled-coil, Silva and co-workers (40) observed that independent folding subdomains with different susceptibility to pressure were evident along its length. When subjected to increased pressure, tropomyosin melted in discrete cooperative blocks along the molecule, and the unfolded domains were likely unstable sites along the coiled-coil chain (40). We examined the hydrophobic clustering in the sequence of rabbit skeletal α-tropomyosin and indeed found nine hydrophobic clusters occupied by 3, 4, 5 consecutive large hydrophobes (I, L, M, V, Y, F) in the core a and d positions, separated by destabilizing residues (A, S, T, Q, D, E, and K) in the hydrophobic core (Fig. 5) (41). The authors of that study proposed that increasing osmotic pressure induced water molecules to infiltrate into the less stable “pressure-sensitive” domains in the hydrophobic core of rabbit skeletal α-tropomyosin, but the more stable, “pressure-insensitive” domains maintain coiled-coil integrity. It is interesting that our clusters of large hydrophobes (Ile, Leu) and small hydrophobes (Ala) could correspond to the pressure-insensitive and pressure-sensitive domains, respectively. Hydrophobic clustering may be an important mechanism for long native coiled-coil proteins to maintain chain integrity yet still accommodate the burial of polar and charged residues to control stability and facilitate different biological functions. Regardless of the length of the coiled-coil, hydrophobic clusters can serve as “knots” to keep the chain together while allowing flexible regions for function.

Hydrophobic clusters control protein stability and are perhaps an evolutionary feature for native proteins to incorporate structurally important stabilizing regions, as well as less stable and more flexible functional domains in a single protein fold.

**DISCUSSION**

Hydrophobic interactions contribute significantly to protein stability because the burial of non-polar surface area is thermodynamically favorable in aqueous solution, and this study has shown that the hydrophobic stabilization is context-dependent. In the coiled-coil model, the additional cluster of three large non-polar residues in P3 enhances the folding of secondary structure and protein stability when compared with P2, where this cluster is missing. Both the three-clustered P3 and the two-clustered P2 peptides have the same inherent hydrophobicity (6 Leu, 3 Ile, and 7 Ala residues in the hydrophobic core), yet their folding and stability differ dramatically. P3 with three hydrophobic clusters is a native protein-like two-stranded coiled-coil with a cooperative unfolding transition. In contrast, P2 with two hydrophobic clusters is only partially folded and significantly less stable when compared with P3. Furthermore, P3 coiled-coil showed a single unfolding transition in DSC, whereas the unfolding transition was not measurable with P2 under identical experimental conditions. Thus, the disruption of the hydrophobic cluster in P2 drastically decreases the enthalpy component of hydrophobic stabilization, which would affect the packing of the two interacting α-helices. The deconvolution of the entropic and the enthalpic components of hydrophobic-residue mutations, i.e. Leu to Ala mutation, is still controversial. For example, Dürr and Jellesarov (37, 38) proposed that the hydrophobic destabilization of a Leu to Ala substitution is largely entropic at room temperature but becomes increasingly enthalpic at higher temperature.

**Hydrophobic Clusters Affect Protein Stability**

**REFERENCES**

1. H. W. G. and D. E. M. (1990) J. Biol. Chem. 265, 14,168-14,173.
2. T. H. and J. E. (1991) J. Mol. Biol. 216, 1087-1091.
3. M. J. and K. T. (1992) Biochemistry 31, 10,434-10,439.
4. S. A. and R. S. (1993) Proc. Natl. Acad. Sci. USA 90, 9,623-9,627.
The destabilizing cluster regions of a coiled-coil may be involved in conformation change that allows for protein-protein interactions. For example, the less stable core regions of tropomyosin could be more easily disrupted for interactions with other proteins (members of the troponin complex and actin involved in muscle regulation) (42). Another example of the importance of flexibility in coiled-coils is the so-called “spring-loaded” mechanism for viral fusion by the hemagglutinin protein of influenza (43), which allows viral entry into host cells. Conformational change in coiled-coil domains has been implicated, for example, in kinesin, kinesin-like proteins, dynein motor proteins, intermediate filament proteins, and tropomyosins (44–50). In addition to mediating protein interactions, hydrophobic residues in the coiled-coil core have been known to control oligomerization state in de novo designed coiled-coils (9–11) and maintain chain orientation in GCN4 by an Asn-Asn 11) and maintain chain orientation in GCN4 by an Asn-Asn