Stabilizing and Destabilizing Clusters in the Hydrophobic Core of Long Two-stranded α-Helical Coiled-coils*

Received for publication, January 30, 2004, and in revised form, March 8, 2004
Published, JBC Papers in Press, March 11, 2004, DOI 10.1074/jbc.M401074200

Stanley C. Kwok and Robert S. Hodges‡
From the Department of Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262

Detailed sequence analyses of the hydrophobic core residues of two long two-stranded α-helical coiled-coils that differ dramatically in sequence, function, and length were performed (tropomyosin of 284 residues and the coiled-coil domain of the myosin rod of 1086 residues). Three types of regions were present in the hydrophobic core of both proteins: stabilizing clusters and destabilizing clusters, defined as three or more consecutive core residues of either stabilizing (Leu, Ile, Val, Met, Phe, and Tyr) or destabilizing (Gly, Ala, Cys, Ser, Thr, Asn, Gln, Asp, Glu, His, Arg, Lys, and Trp) residues, and intervening regions that consist of both stabilizing and destabilizing residues in the hydrophobic core but no clusters. Subsequently, we designed a series of two-stranded coiled-coils to determine what defines a destabilizing cluster from 3 to 7 residues to determine the length effect of the destabilizing cluster on protein stability. The results showed a dramatic destabilization, caused by a single Leu to Ala substitution, on formation of a destabilizing cluster to 5 or 7 hydrophobic core residues in length had little effect on stability (ΔTm of 1.4–2.8 °C). These results suggested that the contribution of Leu to protein stability is context-dependent on whether the hydrophobe is in a stabilizing cluster or its proximity to neighboring destabilizing and stabilizing clusters. To decipher the problem of protein folding, a fundamental understanding of the stability contributions of non-covalent stabilizing and destabilizing interactions is needed. These interactions are encoded in the primary amino acid sequence and guide the initial hydrophobic collapse in an aqueous environment leading to a compact protein fold with well defined secondary structures. The Levinthal’s Principle (1) infers that the protein folding process is not a random and tedious sampling of all possible conformational space, but rather specific pathways are initiated by native (2) and non-native (3) hydrophobic clusters of non-polar amino acids that maintain residual protein interactions even in experimental denaturing environments

*This work was supported in part by the Protein Engineering Network of Centers of Excellence at the University of Alberta, the University of Colorado Health Sciences Center, National Institutes of Health Grant R01GM 61855, and the John Stewart Chair in Peptide Chemistry (to R. S. H.).
†Supported by studentships from the National Science and Engineering Research Council of Canada, Alberta Heritage Foundation for Medical Research and funding from the Canadian Institutes of Health Research.
§To whom correspondence should be addressed. Tel.: 303-315-8837; Fax: 303-315-1153; E-mail: robert.hodges@uchsc.edu.

α-Helical assembly motifs are abundant in proteins (7) and we have focused on what is probably the most widespread assembly motif, the dimeric α-helical coiled-coil, as a model to study protein folding and stability. The two-stranded coiled-coil is an ideal model for such studies because its rod-like nature makes the folding problem a one-dimensional problem, thereby removing much of the complexity found in globular proteins (8). Because this protein motif is widespread in nature (it was estimated that ~3% of all helical sequences are coiled-coils (9)), the coiled-coil and the α-helix have served extensively as models for the study of interactions in proteins, for example, hydrophobic interactions (10–15), ionic interactions (16–20), chain orientation (21–23), chain length effects (24),1 secondary structure propensities (25–29), context dependent interactions such as helix capping motifs (30–33), and clustering of residues in the hydrophobic core (34, 35). Our basic approach is to use de novo designed coiled-coils to isolate specific interactions and test their significance to protein folding and stability.

Coiled-coils are characterized by a heptad repeat motif (gabcdef) in which residues in hydrophobic core positions a and d are the primary determinant of folding and stability (13, 14). Therefore, the hydrophobic cores of short coiled-coils (<40 residues) are often highly enriched with bulky non-polar amino acid residues with few charged or polar residues. In contrast, sequence analysis of longer natural coiled-coil domains, such as tropomyosin (284-residues per polypeptide chain), showed that its core regions are more accommodating of small non-polar, polar, or charged residues that make up ~40% of the core residues (35). Tropomyosin is an ideal protein for sequence analysis because it is a widely distributed, sequence-conserved 100% α-helical coiled-coil with a continuous 3–4 or 4–3 hydrophobic repeat void of any helical stutters, stammers, or breaks (36). When the tropomyosin core a and d residues are represented as a linear array, the continuity of large hydrophobes is often interrupted by small hydrophobic, polar or charged residues creating alternating groupings of what we describe as stabilizing and destabilizing clusters. Based on an experimental coiled-coil stability scale from Hodges and co-workers (13, 14), who substituted all 20 natural amino acid residues into positions a and d in model coiled-coils, we classified Leu, Ile, Val, Met, Phe, and Tyr as stabilizing residues and the remaining 13 amino acids as destabilizing residues. Pro is not found in

1 S. C. Kwok and R. S. Hodges, Biopolymers, submitted for publication.
coiled-coils because of its disruption of a-helical structure. Having defined the criteria of both destabilizing and stabilizing clusters based on free energy measurements, our laboratory described a detailed statistical survey of 34 full-length (284-residue) tropomyosins including different isoforms and 25 different species (35). The distribution of bulky, non-polar stabilizing residues versus destabilizing residues was not random, but rather a cluster of more than 30 hydrophobic core residues was interspersed with clusters of alanine, hydrophilic polar or charged residues, creating alternating clusters of stabilizing and destabilizing residues within the protein (35). In addition, despite the overall sequence homology among tropomyosin molecules, core residues within these clusters are even more conserved than those in non-cluster positions, suggesting that the cluster organization of core residues was perhaps conserved to provide critical structural and functional roles. In support of this concept that alternating regions of stability exist along the coiled-coil, a recent study involving a pressure-induced denaturation experiment of tropomyosin showed that there are discrete domains of stability and instability along the tropomyosin molecule (37).

Despite the many truncation and sequence comparative studies on the tropomyosin molecule aimed to identify folding nucleation sites and critical regions for stability and function (36, 38–40), there is no clear understanding of the relative contribution that stabilizing and destabilizing clusters have on protein stability and what constitutes a destabilizing cluster. We speculate that the phenomenon of destabilizing and stabilizing clusters mediates important structural and functional roles in long native coiled-coils. In particular, the stabilizing clusters could maintain the inherent structure while allowing the less stable and more flexible regions to be involved in function. Our goal in this article was first to extend the sequence analysis of the distribution of stabilizing and destabilizing clusters to longer coiled-coils (>1000 residues), comparing the results of tropomyosin and the myosin rod, and then model the cluster interactions in de novo designed coiled-coils, which have been shown to be an excellent template (41–43) to evaluate folding and stability questions. We designed three series of coiled-coils to determine what defines a destabilizing cluster and varied the length of the destabilizing cluster from 3 to 7 residues in the hydrophobic core to determine the length effect of the destabilizing cluster on overall stability.

EXPERIMENTAL PROCEDURES

Peptide Synthesis and Purification—Detailed peptide synthesis protocols have been described previously (34, 44). The general approach was to use automated solid-phase methodology with conventional t-butyloxycarbonyl chemistry (45). The nascent polypeptide chain was synthesized on copoly(styrene, 1% divinylbenzene)-4-methylbenzhydrylamine-HCl resin, 100–200 mesh, substitution of 0.73 mmol of amino groups per gram (Novabiochem, Switzerland) with appropriate side chain-protected amino acids. Activation reagent O-benzotriazol-1-yl-1,1,3,3-tetramethyluronium hexafluorophosphate (0.45 μ) were dissolved in dichloromethane and N,N-dimethylformamide. Removal of the t-butyloxycarbonyl group was achieved with 50% trifluoroacetic acid in dichloromethane and the completed peptides were released from the 4-methylbenzhydrylamine resin support via hydrogen fluoride cleavage. The peptides were extracted from the resin with glacial acetic acid and dried overnight via lyophilization to yield the crude peptides. The crude peptides were purified by reversed-phase high-performance liquid chromatography (46) on a Zorbax semi-preparative SB-C8 column (250 × 9.4 mm inner diameter, 5 μm particle size, 300-A pore size) by linear AB elution (increasing 0.2% B per min), where eluent A is 0.05% aqueous trifluoroacetic acid and eluent B is 0.05% trifluoroacetic acid in acetonitrile. Analytical reversed-phase high-performance liquid chromatography was carried out to ensure purity and homogeneity of the peptide on a 4.6-mm inner diameter × 150-mm Zorbax SB-C8 column using a linear AB gradient (1% B per min) with the above eluents, and the final product was verified by quantitative amino acid analysis (Beckman model 6300 amino acid analyzer) and by electrospray mass spectrometry on a Mariner Bio- spectrometry work station (Applied Biosystems, Foster City, CA). Formation of the disulfide-bridged homo two-stranded coiled-coils were obtained by overnight stirring in a 100 μM NH4HCO3 buffer, pH 8.5, lyophilized, dissolved in aqueous trifluoroacetic acid and the desired products were purified by analytical reversed-phase high-performance liquid chromatography.

Analytical Ultracentrifugation Equilibrium Experiments—Sedimentation equilibrium analyses were performed on a Beckman XLA analytical ultracentrifuge with absorbance optics at 274 nm for the detection of tyrosine; a detailed experimental procedure and data fitting protocols were described previously (34). To summarize, diazylated peptide samples were loaded onto 12-mm Epon cells at concentrations ranging from 50 to 500 μM. The samples were then spun at 25 °C at 20,000, 25,000, and 35,000 × g for 24 h to achieve equilibrium, and then successive radial absorbance scans were recorded. Peptide molecular weights and oligomerization behavior were 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) fitted to theoretical partial specific volumes based on compositions (47). The partial specific volume of the sample and density of the buffer were calculated using SedNet (version 1.06, University of New Hampshire) using the weighted average of the amino acid content. To obtain oligomeric data, high-shear ultracentrifugation analysis data were converted to linear plots of ln(A) versus r2 (radius) to the theoretical monomer, dimer, and trimer single-species models using the following equation,

\[ C_r = C_m \exp(\frac{r}{r_m})\rho_0 w(r^2 + r_m^2)2RT \]  

where \( C_r \) is the concentration at radius \( r \), \( C_m \) is the concentration at the meniscus, \( m \) is the monomer molecular weight, \( \rho_0 \) is the partial specific volume from SedNet, \( w \) is the buffer density, and \( \omega \) is the angular velocity. All the coiled-coils in this study were found to fit best to the single species monomer model corresponding to the disulfide-bridged two-stranded coiled-coil molecule.

Circular Dichroism Spectroscopy—Circular dichroism (CD) spectroscopy was performed on a Jasco-810 spectropolarimeter with constant N2 flushing (Jasco Inc., Easton, MD) for the determination of secondary structural content and stability (48). A Lauda circulating water bath was used to control the temperature of the optic cell chamber, where rectangular cells of 1-mm path length was used. The concentrations of peptide stock solutions were determined by absorbance at 274 nm in 6 ml media (extinction coefficient, \( \epsilon = 1420 \text{ cm}^{-1} \text{ M}^{-1} \)), one tyrosine per peptide chain) and verified by amino acid analysis. For wavelength scan analyses, 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. Mean residue molar ellipticity was calculated using the equation,

\[ [\theta] = [\theta]_{	ext{ave}} \text{mrw/} 10\text{deg} \]  

where \([\theta]_{	ext{ave}} \) is the observed ellipticity in millidegrees, mrw is the mean residue molecular weight, \( l \) is the optical path length of the CD cell (cm), and \( [\theta] \) is the peptide concentration (mg/ml). Each peptide spectrum was the average of eight wavelength scans collected at 0.1-nm intervals from 195 to 250 nm. The uncertainty in the molar ellipticity values was ±300 deg cm\(^{-1}\) dmol\(^{-1}\).

Temperature-induced Denaturation Monitored by Circular Dichroism—Protein stability measurements were monitored at a wavelength of 222 nm (indicative of a-helical secondary structure) by thermal denaturation. For thermal melting experiments, data points were taken at 1 °C intervals at a scan rate of 60 °C per h. The temperature dependence of the mean residue ellipticity, \( \theta \), was fitted to obtain the fraction of the unfolded state, \( \theta_{\text{U}} \), using a non-linear least-squares algorithm assuming a two-state unfolding reaction with pre-transition (folded state, \( \theta_{\text{H}} \)) and post-transition (unfolded state, \( \theta_{\text{U}} \)) baseline corrections (49),

\[ \theta = \theta_0 \left[ \ln \left( 1 - \frac{\theta_{\text{U}}}{\theta_{\text{H}}} \right) + \frac{-\theta_{\text{H}}}{\theta_{\text{U}}} \right] \]  

where the pre- and post-transition baseline corrections were assumed to be linearly dependent on temperature. The assumption of the two-state unfolding transition of coiled-coils was recently discussed by Dragan and Privalov (50). In thermal denaturation experiments, coiled-coils have often shown a small decrease in ellipticity prior to and after the large unfolding transition associated with coiled-coil unfolding. These


The Effect of a Destabilizing Cluster on Protein Stability

**Cluster Analyses of Tropomyosin and Myosin Sequences**

Comparative analyses of tropomyosin sequences have been reviewed extensively in the literature (36), and the occurrence of Ala residues in destabilizing clusters in tropomyosin observed by our laboratory (35) was especially relevant to the present research. The goal of this study was to examine and compare the number and size of stabilizing and destabilizing clusters and intervening regions in long coiled-coils. The intervening regions do not involve clusters of stabilizing or destabilizing residues. We chose two representative coiled-coils for this analysis: tropomyosin of 284 residues per polypeptide chain and the domain of class II myosin heavy chain, an actin-based molecular motor that hydrolyzes ATP and converts chemical energy into mechanical force for movement (54–56), with >1086 residues per polypeptide chain in the coiled-coil domain. Because of its role as a motor protein, multiple copies of myosin genes are present in all organisms, and the conventional class II myosin is a highly conserved ubiquitous molecule with only short stretches of sequence diversity responsible for specific functions, thus making it useful for sequence analysis.

**Stabilizing and Destabilizing Clusters in Coiled-coils**—In human skeletal myosin, 310 residues are involved in the hydrophobic core (a and d positions), of which 130 are destabilizing residues and 180 are stabilizing residues, based on our classification of stabilizing residues as Leu, Ile, Val, Met, Phe, and Tyr and destabilizing residues as Gly, Ala, Ser, Thr, Asn, Gln, Asp, Glu, Cys, Trp, His, Arg, and Lys. Interestingly, these residues were frequently grouped into stabilizing and destabilizing clusters that we defined as 3 or more consecutive residues in the a and d positions of either stabilizing or destabilizing residues. Our analysis of myosin resulted in the identification of 26 hydrophobic stabilizing clusters and 14 destabilizing clusters, representing 50.6% of the total number of core residues (Table I). In comparison, 74.4% of core residues in tropomyosin were organized into 9 stabilizing clusters and 6 destabilizing ones (Table I). Although the coiled-coil domain of myosin is nearly four times longer than in tropomyosin, the total numbers of stabilizing and destabilizing residues as a percentage of the total residues in the hydrophobic core were remarkably similar; 58.1% in myosin and 58.5% in tropomyosin were stabilizing residues, and correspondingly, the destabilizing residue composition was 41.9% in myosin and 41.5% in tropomyosin (Table I). Furthermore, the percentage of destabilizing and stabilizing clusters compared with the total number of clusters was very similar. Myosin has 14 destabilizing clus-

---

**Table I**

| Human skeletal myosin | Human skeletal tropomyosin |
|-----------------------|-----------------------------|
| **Total a and d positions** | 310 | 82 |
| Total destabilizing residues | 130 (41.9%) | 34 (41.5%) |
| Total stabilizing residues | 180 (58.1%) | 48 (58.5%) |
| **Total number of clusters and intervening regions a** | 62 | 22 |
| Total number of clusters | 40 (65%) | 15 (68%) |
| Number of destabilizing clusters | 14 (35%) | 6 (40%) |
| Number of stabilizing clusters | 26 (65%) | 9 (60%) |
| Total number of intervening regions a | 22 (35%) | 7 (32%) |
| Total number of destabilizing residues in destabilizing clusters | 59° | 23 |
| Number of residues b | Ala 22 (44%) | Ala 17 (74%) |
| Thr 5 (10%) | Ser 3 (13%) |
| Total number of stabilizing residues in stabilizing clusters | 107° | 38 |
| Number of residues b | Leu 50 (47%) | Leu 21 (55%) |
| Ile 24 (22%) | Ile 6 (18%) |
| Val 16 (15%) | Val 5 (13%) |
| Met 11 (10%) | Tyr 4 (10%) |
| Total number of destabilizing residues in intervening regions | 79° | 11 |
| Number of residues b | Lys 21 (27%) | Ala 6 (55%) |
| Ala 10 (13%) | Gln 2 (18%) |
| Glu 10 (13%) | Ser 8 (10%) |
| Thr 8 (10%) | |
| Total number of stabilizing residues in intervening regions | 71° | 10 |
| Number of residues b | Leu 47 (68%) | Leu 4 (40%) |
| Val 11 (15%) | Met 3 (30%) |
| Ile 2 (20%) | Tyr 1 (10%) |

---

a Intervening regions within the hydrophobic core (a and d positions) that do not contain destabilizing or stabilizing clusters.

b Only the residues that occur at greater than 10% are listed.

c Two stabilizing residues are involved in frameshift regions; one destabilizing residue is involved in a frame shift region.

---

small decreases were attributed to concentration-independent transitions because of end fraying and other molecular motion that does not lead to helix unfolding (50). These observations were accounted for in our data set by the pre- and post-transition baselines. The calculated fraction of the unfolded state, \( f_{Uexp} \), was used to determine \( \Delta G \) (51),

\[
f_{Uexp} = \exp\left(-\frac{\Delta G_{app}}{RT}\right)\left[1 + \exp\left(-\frac{\Delta G_{app}}{RT}\right)\right]
\]

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

\[
\Delta G_{app} = \Delta H_{app}(1 - e^{\Delta C_p/T}) + \Delta C_p(T - T_m) - R\ln(\theta/T_m)
\]

where \( T \) is the observed temperature, \( T_m \) is the temperature midpoint of the thermal transition, \( \Delta H_{app} \) is the apparent enthalpy of unfolding, and \( \Delta C_p \) is the change in heat capacity change associated with protein unfolding. \( \Delta C_p \) and \( \Delta H \) were assumed to be constant within the temperature range (52, 53). These thermodynamic parameters were fitted using the program SigmaPlot, and the derived fraction folded parameters were used to determine an apparent free energy stability term, \( \Delta G_{app,20} \), using the following equation,

\[
\Delta G_{app,20} = -R\ln\left[\frac{f_{Uexp}}{f_{Uexp}/f_C - 1}\right]
\]

and then plotting the fraction folded data and extrapolating to 20 °C.

**RESULTS**

---

Comparative analyses of tropomyosin sequences have been reviewed extensively in the literature (36), and the occurrence of Ala residues in destabilizing clusters in tropomyosin observed by our laboratory (35) was especially relevant to the present research. The goal of this study was to examine and compare the number and size of stabilizing and destabilizing clusters and intervening regions in long coiled-coils. The intervening regions do not involve clusters of stabilizing or destabilizing residues. We chose two representative coiled-coils for this analysis: tropomyosin of 284 residues per polypeptide chain and the domain of class II myosin heavy chain, an actin-based molecular motor that hydrolyzes ATP and converts chemical energy into mechanical force for movement (54–56), with >1086 residues per polypeptide chain in the coiled-coil domain. Because of its role as a motor protein, multiple copies of myosin genes are present in all organisms, and the conventional class II myosin is a highly conserved ubiquitous molecule with only short stretches of sequence diversity responsible for specific functions, thus making it useful for sequence analysis.

**Stabilizing and Destabilizing Clusters in Coiled-coils**—In human skeletal myosin, 310 residues are involved in the hydrophobic core (a and d positions), of which 130 are destabilizing residues and 180 are stabilizing residues, based on our classification of stabilizing residues as Leu, Ile, Val, Met, Phe, and Tyr and destabilizing residues as Gly, Ala, Ser, Thr, Asn, Gln, Asp, Glu, Cys, Trp, His, Arg, and Lys. Interestingly, these residues were frequently grouped into stabilizing and destabilizing clusters that we defined as 3 or more consecutive residues in the a and d positions of either stabilizing or destabilizing residues. Our analysis of myosin resulted in the identification of 26 hydrophobic stabilizing clusters and 14 destabilizing clusters, representing 50.6% of the total number of core residues (Table I). In comparison, 74.4% of core residues in tropomyosin were organized into 9 stabilizing clusters and 6 destabilizing ones (Table I). Although the coiled-coil domain of myosin is nearly four times longer than in tropomyosin, the total numbers of stabilizing and destabilizing residues as a percentage of the total residues in the hydrophobic core were remarkably similar; 58.1% in myosin and 58.5% in tropomyosin were stabilizing residues, and correspondingly, the destabilizing residue composition was 41.9% in myosin and 41.5% in tropomyosin (Table I). Furthermore, the percentage of destabilizing and stabilizing clusters compared with the total number of clusters was very similar. Myosin has 14 destabilizing clus-
ters (35%) and 26 stabilizing clusters (65%). In comparison, tropomyosin has 6 destabilizing clusters (40%) and 9 stabilizing clusters (60%) (Table I).

Not surprisingly, the most popular residue in stabilizing clusters in both myosin and tropomyosin was Leu (~50%) (Table I), which agrees with Leu being found most frequently in the core of coiled-coils (57, 58). Interestingly, the destabilizing clusters in myosin and tropomyosin consisted mainly of Ala, where Ala was present in all six of the destabilizing clusters in tropomyosin and in 12 of 14 of the destabilizing clusters in myosin (Tables II and III). The high frequency of occurrence of Ala in the destabilizing cluster of myosin (44%, Table I) and tropomyosin (74%, Table I) is understandable considering the properties of Ala. Ala is the smallest non-polar amino acid side chain, with the highest α-helical propensity of amino acid side chains (26, 28) and generally provides more stability to coiled-coils than any other residue classified as destabilizing (13, 14).

Thus, Ala allows for destabilization of the hydrophobic core but at the same time maintains the α-helical structure through its high α-helical propensity.

The size of hydrophobic clusters in tropomyosin varied from 3 to 5 residues, whereas in myosin the hydrophobic stabilizing clusters varied from 3 to 6 consecutive hydrophobes with the cluster length of 5 being most prevalent in tropomyosin and the cluster length of 4 being most prevalent in myosin (Tables II and III). Although the destabilizing cluster length in tropomyosin and myosin varied from 3 to 7 consecutive destabilizing residues, a cluster length of 3 destabilizing residues was most common in both proteins (Tables II and III).

Intervening Regions in Coiled-coils—We have defined intervening regions within the hydrophobic core (a and d positions) as regions that do not contain destabilizing or stabilizing clusters. There are 22 intervening regions in the myosin hydrophobic core containing 71 stabilizing residues and 79 destabilizing residues. In tropomyosin the number of intervening regions is 7 containing 10 stabilizing residues and 11 destabilizing residues. The ratio of stabilizing to destabilizing residues in intervening regions of both proteins, even through myosin is ~4 times longer, is the same and close to 1:1. Intervening regions in myosin varied in length from a single residue to as long as 27 hydrophobic core residues. However, 20 of the intervening regions varied from 2 to 13 hydrophobic core residues in length with 1 and 27 occurring only once. Intervening regions in tropomyosin vary in length from 1 to 7 hydrophobic core residues. Fig. 1 shows a map of the stabilizing and destabilizing clusters and intervening regions throughout the sequences of both proteins. The percentage of intervening regions to clusters was similar in both proteins: intervening regions 35% in myosin and 32% in tropomyosin, with clusters making up 65% in myosin and 68% in tropomyosin (Table I). What was different between the two proteins was that myosin has 48% of the hydrophobic core residues found in intervening regions, whereas tropomyosin has only 26%. This difference is because of myosin having larger intervening regions than tropomyosin (Fig. 1). The stabilizing and destabilizing clusters and intervening regions were well distributed throughout the sequences of the two coiled-coils (Fig. 1). In tropomyosin the dominating destabilizing residue found in destabilizing clusters and intervening regions was Ala (Table I). In contrast, in myosin the dominating destabilizing residue found in the destabilizing clusters was Ala but in the intervening regions the dominating residue found in the hydrophobic core was Lys (Table I). The dominating stabilizing residue found in the stabilizing clusters and intervening regions was Leu in both myosin and tropomyosin (Table I).

Coiled-Coil Design—We designed a series of two-stranded
parallel \( \alpha \)-helical coiled-coils with different lengths (5, 6, and 7 heptads), all containing a N-terminal and C-terminal hydrophobic cluster (the C-terminal cluster has three consecutive core positions, whereas the N-terminal cluster has four consecutive core positions occupied by stabilizing large aliphatic hydrophobes, Ile and Leu at positions \( a \) and \( d \), respectively) (Fig. 2). Ile and Leu were shown to be major stabilizing residues in the hydrophobic core \( a \) and \( d \) positions relative to Ala (10, 11,}

### Occurrence of clusters in the hydrophobic core of tropomyosin

| Cluster size | Sequence Positions | Human skeletal tropomyosin \( \alpha \) chain coiled-coil (residues 1–284)\(^a\) |
|-------------|-------------------|----------------------------------|
| 3           | 57, 60, 64        | LYL 74, 78, 81                   |
|             | 267, 270, 274     | YIL 116,120, 123                 |
|             |                   | 235,238, 242                     |
| 4           | 1, 4, 8, 11       | MIML 179,183,186,190             |
|             | 39, 43, 46, 50    | LLLL AAS                         |
|             | 221, 225, 228, 232| YILL                             |
| 5           | 85, 88, 92, 95, 99| VLLVL                            |
|             | 162, 165, 169, 172, 176| YVLIL                        |
|             | 193, 197, 200, 204, 207 | LLVLL                           |
|             | 246, 249, 253, 256, 260 | VLILL                           |
| 7           | 15, 18, 22, 25,29,32,36 | KAAAKAS                       |

\(^a\) Swiss-Prot accession number P09493.  
A stabilizing or destabilizing cluster is defined as 3 or more consecutive residues in the \( a \) and \( d \) positions of either stabilizing residues (Ile, Leu, Val, Met, Phe, and Tyr) or destabilizing residues (Gly, Ala, Cys, Ser, Thr, Asn, Gln, Asp, Glu, His, Lys, Arg, and Trp). Stabilizing and destabilizing residues are based on the results of Wagschal et al. (13) and Tripet et al. (14).
The Effect of a Destabilizing Cluster on Protein Stability

| Peptide Name | Heptad Code | Amino Acid Sequences |
|--------------|-------------|---------------------|
| 5A1          | αβα         | Ac-EIEALK-AKLAK-EAK-|
|              |             | -GAE-LH-GC-GC-y-am  |
| 5A3          | αβα         | Ac-EIEALK-AKLAK-EAK-|
|              |             | -GAE-LH-GC-GC-y-am  |
| 6A1          | αβα         | Ac-EIEALK-AKLAK-EAK-|
|              |             | -GAE-LH-GC-GC-y-am  |
| 6A3          | αβα         | Ac-EIEALK-AKLAK-EAK-|
|              |             | -GAE-LH-GC-GC-y-am  |
| 6A5          | αβα         | Ac-EIEALK-AKLAK-EAK-|
|              |             | -GAE-LH-GC-GC-y-am  |
| 7A1          | αβα         | Ac-EIEALK-AKLAK-EAK-|
|              |             | -GAE-LH-GC-GC-y-am  |
| 7A3          | αβα         | Ac-EIEALK-AKLAK-EAK-|
|              |             | -GAE-LH-GC-GC-y-am  |
| 7A5          | αβα         | Ac-EIEALK-AKLAK-EAK-|
|              |             | -GAE-LH-GC-GC-y-am  |
| 7A7          | αβα         | Ac-EIEALK-AKLAK-EAK-|
|              |             | -GAE-LH-GC-GC-y-am  |

Fig. 2. Peptide nomenclature, sequences, and schematics of the coiled-coil analogs used in this study. Top panel shows the three series of coiled-coil proteins, 5A1 and 5A3; 6A1, 6A3, and 6A5; and 7A1, 7A3, 7A5, and 7A7, which are denoted by the heptad length (5, 6, and 7) and by the length of the alanine cluster (A1 to A7). For example, 6A5 refers to a six-heptad coiled-coil with a 5-alanine cluster. Each analog is also named by the arrangement of its constituent heptad repeats described by the heptad code. Three different heptads were used (α, EIEALK; β, KAEALEG; γ, KAEEAEGL). The alanine destabilizing cluster size is increased by replacing γ-heptads for β-heptads that is a single Leu to Ala substitution, which is denoted by the rectangle box (e.g. Leu in 5A1 is replaced by an Ala in 5A3). The heptad repeat is denoted as gabcdef, which is shown above the first heptad of the coiled-coils. The residues at positions a and d in the hydrophobic core are bold. 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). The boxes indicate three or more consecutive Ala residues in the hydrophobic core. Ac, N\(^{\text{Ac}}\)-acetyl; am, C-terminal amide.

13, 14). Also, Ile at position a and Leu at position d were shown to favor the two-stranded coiled-coil over higher-order oligomers (59). In addition, these residues were also the most common ones in the stabilizing clusters of myosin and tropomyosin, making up 70% (Table I). The α-heptad Glu-Ile-Glu-Ala-Leu-Lys-Ala was used at both ends of all coiled-coils. This provided in addition to the stable hydrophobic core at positions a and d, interchain and intrachain ionic interactions from Lys and Glu residues at positions b, e, and g, resulting in ionic stabilization because of interchain electrostatic attractions (i to i’ + 5 or g to e’) and intrachain ionic attractions (i to i + 3 or i to i + 4) (16, 17). Ala at positions c and f provided excellent helical propensity, further increasing stability (26–28). As described previously, we introduced a C-terminal disulfide bridge, Gly-Gly-Cys-Tyr linker, to facilitate the formation of a parallel and in-register coiled-coil (34) and the single Tyr residue per polypeptide chain allowed for protein concentration determination by UV spectroscopy. The disulfide bridge also removed the monomer-dimer equilibrium observed in two-stranded coiled-coils, making the comparisons of coiled-coil stability concentration independent.

Based upon the above analyses, we chose to incorporate Ala as a destabilizing residue in the hydrophobic core of these coiled-coils. The additional advantage of choosing Ala as a destabilizing residue is that it does not introduce any context-dependent interactions such as hydrogen bonding or electrostatic effects that may occur with polar or charged side chains. In Fig. 2, we have boxed the Leu to Ala substitutions that created 3-, 5-, and 7-residue Ala clusters in the hydrophobic core of these coiled-coils. For example, a single Leu to Ala substitution in peptide 7A1 created a 3-Ala cluster in 7A3; a single Leu to Ala substitution in 7A3 created a 5-Ala cluster in 7A5; and a single Leu to Ala substitution in 7A5 created a 7-Ala cluster in 7A7. Otherwise the peptides were of identical length and sequence.

We designed three versions of two-stranded parallel α-helical coiled-coils with different heptad lengths (5, 6, and 7 heptads denoted as peptides 5, 6, and 7). The number of residues ranged from 39 to 53 residues. The coiled-coils differed in the number of consecutive Ala residues in the hydrophobic core from 1, 3, 5, and 7 (denoted A1, A3, A5, and A7). Thus, peptide 5A3 meant the peptide contained 5 heptads with 3 consecutive Ala residues in the hydrophobic core (Fig. 2). We chose different coiled-coil lengths to see if a Leu to Ala substitution was affected by parallel and in-register coiled-coil (34) and the single Tyr residues in the hydrophobic core. For example, a single Leu to Ala substitution in peptide 7A1 created a 3-Ala cluster in 7A3; a single Leu to Ala substitution in 7A3 created a 5-Ala cluster in 7A5; and a single Leu to Ala substitution in 7A5 created a 7-Ala cluster in 7A7. Otherwise the peptides were of identical length and sequence.
tion \(a\) instead of Ile. In addition, Gly was chosen to replace Ala at position \(f\), which has a much lower helical propensity than Ala (26, 28) and by switching the Lys at \(g\) and the Glu at \(e\) we maintained the same interchain electrostatic attractions \(i\) to \(i' + 5\) but introduced \(i\) to \(i' + 4\) and \(i\) to \(i' + 3\) intrachain repulsions with the Glu residue at position \(b\) that further destabilized the heptad (17–20, 23). Thus, peptides 5A1, 6A1, and 7A1 consisted of two, three, and four \(\beta\)-heptads, respectively. The helical propensity effect was estimated to be \(\sim 0.5\) kcal/mol (60) for each Ala to Gly substitution and 0.2 kcal/mol for the added repulsions (61) in each chain per heptad compared with the Ile to Ala substitution at position \(a\) of 1.95 kcal/mol for each substitution when made in the center of a hydrophobic cluster or 3.9 kcal/mol for this substitution in a homodimeric coiled-coil (13, 14). This suggests that the \(\beta\)-heptad could be as much as 5.3 kcal/mol less stabilizing than the \(\alpha\)-heptad in a homodimeric coiled-coil (3.9 + 2\(\times\)0.5 + 2\(\times\)0.2 = 5.3 kcal/mol). Therefore, as the chain length increased from 5 to 7 heptads, the overall coiled-coil stability was expected to decrease. The \(\gamma\)-heptad Lys-Ala-Glu-Ala-Ala-Glu-Gly was identical to the \(\beta\)-heptad with the exception that Ala replaced the Leu in the \(\beta\)-heptad at position \(d\). This substitution was expected to decrease stability by as much as 1.9 kcal/mol for each substitution when made in the center of a hydrophobic cluster or 3.8 kcal/mol in a homodimeric coiled-coil (14). Thus, the \(\gamma\)-heptad could be as much as 3.8 kcal/mol less stabilizing than the \(\beta\)-heptad in a homodimeric coiled-coil. The single Leu to Ala substitution in each chain of the homodimeric coiled-coils used in this study would be expected to be significantly large and equal in all the coiled-coils unless there is a context dependence of the location of the Leu residue. For example, one could ask the question, does the location of a leucine residue next to a destabilizing cluster have a dramatic effect on its contribution to overall stability of the coiled-coil? To minimize any extraneous context-dependent contributions other than the desired interaction under investigation (destabilizing clusters), the Leu to Ala substitutions were made in the center of the coiled-coils (Fig. 2), because these substitution sites should be less susceptible to end-fraying effects (10).

A schematic representation of the coiled-coil 6A5 showed the spatial orientation of the destabilizing cluster and the conceptualization of small alanine residues relative to the larger hydrophobic residues Leu or Ile in the hydrophobic core (Fig. 3, top panel). Inter-chain attractive ionic interactions mediated by charged residues at \(e\) and \(g\) provided additional stabilization across the coiled-coil interface (17–19). A space-filling model of the side chain interactions of Leu-Leu and Ile-Ile pairing in the coiled-coil core (6IL) was modeled into an idealized coiled-coil domain of cortexillin (Fig. 3, bottom panel left). On the right, coiled-coil analog 6A5 with a destabilizing cluster of five consecutive Ala was modeled into the same coiled-coil backbone showing the less than ideal van der Waals contact and spatial gap (Fig. 3, bottom panel). This leaves the hydrophobic core in the Ala cluster much more accessible to H\(_2\)O and competition with hydrogen bonds that stabilize the individual \(\alpha\)-helices and thus an expected destabilization relative to Leu and Ile residues in the hydrophobic core.

**Structural and Biophysical Characterization of Coiled-coils**—For the coiled-coils to be useful models that permit the investigation of a destabilizing cluster effect, they have to be dimeric, fully \(\alpha\)-helical, and exhibit reasonable stabilities when oxidized. The circular dichroism spectra of a representative oxidized coiled-coil, 5A1, showed excellent helicity, \(>97\%\), in benign buffer (50 mM KCl, 100 mM phosphate buffer, pH 7.0, characteristic of physiological pH and ionic strength), and even in the helix-promoting solvent 50% trifluoroethanol (99% helix), no significant helix induction was observed (Fig. 4, Table IV). The ratio of \(\theta\)\(_{222/208}\) is greater than 1 for fully folded coiled-coils in benign buffer and less than 1 for fully folded single-stranded \(\alpha\)-helices in trifluoroethanol (10, 62, 63). In benign buffer all of the coiled-coils showed this characteristic ratio of 1.0 to 1.03 (Table IV). In the presence of 50% trifluoroethanol, the non-covalent hydrophobic interactions between helices were eliminated and thus, the \(\theta\)\(_{222/208}\) dropped to 0.88 to 0.93, representing the fully helical yet single-stranded state. All other model coiled-coils, from 5A1–5A3, 6A1–6A5, and 7A1–7A7, including those with destabilizing Ala clusters, exhibited high helical content (98–99%) in benign buffer. Therefore, the presence of destabilizing clusters in our model coiled-coils did not have any significant effect on the \(\alpha\)-helical content or affect the oligomeric state. We confirmed the dimeric coiled-coil oligomerization state of all analogs by sedimentation equilibrium analyses. Fig. 5 shows the sedimentation equilibrium results from two representative model coiled-coils, 5A1 and
7A5, of different lengths and different size of destabilizing Ala clusters. These coiled-coil analogs showed an excellent correlation to the single species two-stranded disulfide-bridged coiled-coil unit (monomer, M), and no indication of higher oligomeric states (dimer, D or trimer, T) was observed. Observed and theoretical molecular weights are reported in Table IV. Taken together, the circular dichroism and sedimentation equilibrium data showed that all the coiled-coil analogs were in the fully-folded two-stranded α-helical state.

The Effect of a Three-residue Alanine Cluster on Coiled-coil Stability—Having shown that these model coiled-coils are fully folded, we determined their thermal stabilities by circular dichroism monitored at \([\Theta]_{222}\). All three coiled-coils over the temperature range of 5 to 95 °C without a destabilizing cluster (5A1, 6A1, 7A1) exhibited a cooperative two-state unfolding profile with \(T_m\) of 87.3, 79.3, and 71.4 °C, respectively (Table IV). Coiled-coil 5A1 was the most stable of the three as expected based on our design criteria and can be attributed to a greater stability density of the five-heptad coiled-coil relative to the longer coiled-coils. The hydrophobic core density was calculated using the sum of the hydrophobic core residue stability predicted based on our design criteria and can be attributed to a well-defined core density.

The hydrophobic core density was calculated using the sum of the hydrophobic core residue stability predicted based on our design criteria and can be attributed to a well-defined core density. The effect of a destabilizing cluster on protein stability (Fig. 6, panels A and D), as demonstrated by a decrease in the thermal midpoint of 17.1 °C (Table V). Likewise, large drops in protein stability were observed for the creation of this 3-residue Ala destabilizing cluster.

The Effect of a Destabilizing Cluster on Protein Stability

| Peptide | Number of heptads | Number of consecutive alanines | Observed \(M_r\) | Theoretical \(M_r\) | \([\Theta]_{222}\) Benign | \([\Theta]_{222}\) TFE | \(\alpha\)-Helix% | \([\Theta]_{222}/208\) Benign | \([\Theta]_{222}/208\) TFE | \(T_m\) °C | \(\Delta G_{app,20}\) kcal mol\(^{-1}\) |
|---------|------------------|-----------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|--------------|----------------|
| 7A1     | 7                | 1                           | 11,060         | 11,000         | -35,300        | -36,000        | 99             | 99             | 1.03           | 0.91         | 71.4           | 8.6          |
| 7A2     | 7                | 1                           | 11,180         | 10,916         | -33,200        | -34,900        | 94             | 98             | 1.02           | 0.89         | 50.2           | 5.1          |
| 7A3     | 7                | 5                           | 10,960         | 10,832         | -31,700        | -33,500        | 89             | 99             | 1.02           | 0.92         | 48.0           | 5.0          |
| 7A4     | 7                | 7                           | 10,800         | 10,738         | -30,000        | -33,600        | 90             | 97             | 1.01           | 0.91         | 46.6           | 5.1          |
| 6A2     | 5                | 3                           | 9,760          | 9,518          | -33,500        | -34,300        | 96             | 97             | 1.01           | 0.93         | 59.5           | 2.7          |
| 6A3     | 5                | 5                           | 9,500          | 9,434          | -32,300        | -34,200        | 92             | 97             | 1.02           | 0.88         | 56.7           | 2.7          |
| 6A4     | 5                | 4                           | 8,990          | 8,810          | -31,900        | -33,800        | 93             | 99             | 1.01           | 0.91         | 87.3           | 11.4         |
| 5A1     | 5                | 5                           | 8,340          | 8,240          | -33,600        | -34,200        | 98             | 99             | 1.01           | 0.88         | 87.1           | 11.4         |
| 5A2     | 5                | 4                           | 8,100          | 8,080          | -33,400        | -34,200        | 99             | 100            | 1.02           | 0.88         | 87.0           | 11.4         |

\(\Delta G_{app,20}\) is the change in free energy of the coiled-coil extrapolated from the thermal denaturation experiment at 20 °C (see “Experimental Procedures”).
ing cluster (EGG3) in models 6A1–6A3 (Fig. 6, panels D and E) and 7A1–7A3 (Fig. 6, panels C and F) with decreases in thermal midpoints of 19.8 and 21.2 °C, respectively (Table V). This decrease in protein stability was not surprising because it corresponded to a loss of 78 Å² of non-polar surface area, which was predicted to decrease stability by 2.7 to 3.5 kcal/mol (64). We calculated this change in free energy (G) for the creation of a 3-residue Ala destabilizing cluster to range from 2.7 to 3.5 kcal/mol in our models (Table V). In these peptides, the Leu was in the context of a single destabilizing Ala residue on each side in the hydrophobic core, which compared favorably with a Leu-Ala destabilizing contribution of 2.6 kcal/mol reported recently by Lu and Hodges (35) where a Leu to Ala substitution was made in the center of a 3-residue hydrophobic stabilizing cluster surrounded by destabilizing residues (EEE GEG EEE 3 EEEE).

The Effect of Leu to Ala Substitutions Juxtaposed between a Destabilizing Cluster and a Destabilizing Residue—Coiled-coils 6A3 and 7A3 contained a Leu with an adjacent 3-residue alanine cluster on one side and a single destabilizing residue on the other (EEE GEG EEE). The substitution of the Leu with Ala in each case created a 5-residue Ala cluster (EEE GEG EEE 3 EEEE). The question being addressed was the contribution of this leucine residue to protein stability. When we compared the stabilities of 6A3–6A5 and 7A3–7A5 (EEE GEG EEE 3 EEEE), the loss of $T_m$ was marginal, with $\Delta T_m$ of 2.8 and 2.2 °C, respectively (Fig. 6, panels B, E and panels C, F; Table IV). This very small decrease in stability contrasted with the large decrease we observed for the creation of a 3-Ala cluster (EEE GEG EEE) in the three coiled-coil models with different overall stabilities. The creation of a 5-residue Ala cluster did not destabilize the coiled-coil (Table V) despite the inherent identical Leu-Ala substitution.

The coiled-coil 7A5 contained a Leu with an adjacent 5-residue alanine cluster on one side and a single destabilizing residue on the other (EEE GEG EEE 3 EEEE). The substitution of this Leu with Ala created a 7-residue Ala cluster in the coiled-coil (EEE GEG EEE 3 EEEE). Comparison of 7A5 and 7A7 resulted only in a decrease of 1.4 °C in $T_m$ (Fig. 6, panels C and F) with no decrease in stability observed when the $\Delta G$ was calculated (Table V). This observed non-linearity between protein stability and the environment surrounding the leucine residue in the hydrophobic core is critical in understanding protein folding, stability, and function of coiled-coils. These results suggest that the length of a destabilizing cluster can be extremely long without having a significant impact on the overall stability of the coiled-coil. For example, analogs 7A3, 7A5, and 7A7 have 3, 5, and 7 alanine clusters and essentially the same protein stability.

**DISCUSSION**

Sequence analysis of two highly conserved and ubiquitous proteins that differ dramatically in sequence, function, and length of their coiled-coils, tropomyosin (284 residues) and myosin heavy chain domain (1086 residues) showed remarkable similarity in the following ways: first, the percentage of stabilizing and destabilizing residues in the hydrophobic core (~58 and 42%, respectively); second, in both coiled-coils these
residues are organized into stabilizing and destabilizing clusters and intervening regions that are well distributed along the molecules; third, the percentage of each cluster type is 60–65% stabilizing clusters and 35–40% destabilizing clusters in both proteins (Table I); fourth, the percentage of intervening regions to the total number of clusters and intervening regions is 32–35% in both proteins; fifth, the ratio of destabilizing and stabilizing residues in intervening regions is close to 1:1 in both proteins.

The distribution of cluster size in stabilizing clusters and destabilizing clusters is summarized in Fig. 7. In myosin, 88% of the stabilizing clusters are of 3, 4, or 5 consecutive residues
in the hydrophobic core with a predominant cluster size of 4. In tropomyosin 100% of the stabilizing clusters are of 3, 4, or 5 residues with a predominant cluster size of 5. We have previously shown that the minimum size of a stabilizing cluster is 3 consecutive hydrophobic residues in the core (35). In both myosin and tropomyosin, the predominant size of a destabilizing cluster is 3 (Tables II and III) and this agrees with our experimental results.

The dominating residue in the stabilizing clusters in both molecules is Leu (~50%), with Leu and Ile accounting for ~70% (Table I). This is not unexpected because these two residues are the most hydrophobic residues of the aliphatic side chains and can pack extremely well into the hydrophobic core of coiled-coils. The dominating residue in the destabilizing clusters in both molecules is Ala, accounting for 44 and 74% in myosin and tropomyosin, respectively (Table I). This is not surprising when one considers that Ala has the highest \( \alpha \)-helical propensity of the 20 amino acids and, although the least hydrophobic of the non-polar amino acids, Ala is the easiest to pack in the hydrophobic core because it lacks polar or charged character and still provides greater stability than the polar and charged size chains in coiled-coils (14). This suggests that nature may want to destabilize the coiled-coil by using these destabilizing clusters and at the same time maintain the overall \( \alpha \)-helical structure in these regions. The stabilizing and destabilizing clusters and intervening regions are well distributed along the coiled-coils and suggests an evolutionary adaptation for long coiled-coil proteins where structure integrity is maintained by the stabilizing clusters, whereas localized regions of less stability can occur. Obviously these three types of regions control the overall protein stability, provide additional flexibility in certain regions of the coiled-coil for interactions with other molecules, control folding and assembly, or allow transmission of conformational change.

In the “Experimental Procedures,” we have clearly shown that once a destabilizing cluster of three consecutive alanine residues occurs in the hydrophobic core, extension of this cluster to 7 consecutive alanine residues had little effect on \( \alpha \)-helical structure or stability of the coiled-coil. The results shown in Fig. 7 show that the predominant size of a destabilizing cluster in tropomyosin and myosin is 3, in agreement with the experimental results that the major destabilization of the coiled-coils is achieved with a cluster size of 3. Interestingly, a cluster of 7 destabilizing residues was observed in tropomyosin (Table III). This unusual occurrence suggests a functional role to this region of the coiled-coil. Increasing the size of the destabilizing cluster could increase the flexibility of this region without affecting overall stability. Theoretical studies have suggested that local regions of instability along a coiled-coil could cause bubbling and chain separation introducing destabilizing entropy (65, 66). In contrast, short coiled-coils (<35 residues) almost always unfold from the ends because the two termini are most susceptible to end-fraying because of the lack of hydrogen bonding partners in the individual \( \alpha \)-helices of the coiled-coil (67, 68).

Cohen and co-workers (69) have determined the x-ray structure of a fragment of tropomyosin (residues 1–81), which contained the major destabilizing cluster Lys13-Ala14-Ala22-Ala35, Lys39-Ala42-Ser46, which is identical in the human and chicken sequence (Table III). The hydrophobic cluster that follows the destabilizing cluster is also identical in both species involving Leu24-Leu43-Leu46 and Leu49 in the hydrophobic core. Their analysis of the crystal structure in this region was as follows: first, this segment rich in core Ala residues (residues 22–36) was axially out-of-register by ~1.2 Å relative to canonical coiled-coils; second, the coiled-coil in this region was narrower by ~2 Å compared with the hydrophobic cluster of Leu residues that follows the destabilizing cluster; and third, there is a bend of the coiled-coil axis between the destabilizing and stabilizing cluster (in this case at approximately residue 36). The authors speculate that the structural effects of an alanine cluster on the axial register, symmetry, and conformational variability of the two stranded coiled-coil appear to be important for the role of tropomyosin in the regulation of muscle contraction (68). However, it should be noted that from our analysis these same destabilizing clusters occur in myosin where the myosin coiled-coil has a totally different functional role than tropomyosin. Tropomyosin has been shown to bind to actin and to move to a different position on the actin filament during muscle contraction, whereas the myosin coiled-coils are packed together in a staggered fashion to form a myosin filament. This suggests that the common occurrence of stabilizing and destabilizing clusters can have different roles in different coiled-coil proteins.

Singh and Hitchcock-DeGregori (70) recently mutated a 3-Ala cluster in tropomyosin (Ala74, Ala78, Ala81) to Leu74, Val78, Leu81 and showed that this change significantly stabilized tropomyosin but decreased actin affinity by >10-fold. They also made a mutant Gln74, Asn78, Gln81, which had the same stability and affinity to actin as the native protein with Ala74, Ala78, Ala81. This suggests that the important determi-
nant is the destabilizing effect rather than the exact composition of the destabilizing residues in the destabilizing cluster. Clearly, if we are going to understand the relationship of structure and function in coiled-coils, we will have to understand the relative contribution the three regions (stabilizing and destabilizing clusters and intervening regions) make to the stability of local regions and to the overall stability of the protein.

In the native coiled-coils, tropomyosin and myosin, Leu is not only the most abundant residue in the hydrophobic core of stabilizing clusters but also the most abundant stabilizing residue in intervening regions (Table I); furthermore, it is also evenly distributed between positions α and β. Similarly, Ala is the most predominant residue in the hydrophobic core in destabilizing clusters (Table I). Thus, in our model of coiled-coils we used Leu to Ala substitutions to understand the contribution that Leu makes to protein stability in various contexts in the hydrophobic core. For example, a Leu not in a hydrophobic cluster but surrounded by one destabilizing residue on each side and adjacent to a hydrophobic cluster contributes substantially to stability (compare (EEEE) to (GGGG)). The ΔAG values for the Leu to Ala substitution contributed 2.7–3.5 kcal/mol per coiled-coil (Table V). The occurrence of Leu in this context in myosin was significant at 11 times. Our results suggest that in this environment in the hydrophobic core, the Leu residue still contributes significantly to the overall stability of the coiled-coil. However, once the Leu residue is adjacent to a destabilizing cluster on one side and a destabilizing residue on the other (GGGG → EE), the Leu residue contributes essentially nothing to stability (Table V). Interestingly, this situation occurs 6 times in myosin. The question naturally arises as to why Leu residues are found in the hydrophobic core of native coiled-coils if they do not contribute to overall protein stability. This we believe is for the preservation of the diameter and structure of the coiled-coil in a particular region. Results from the earlier described tropomyosin fragment and recent crystallographic evidence on trimeric coiled-coil Lpp-56 both showed that the core diameter of the coiled-coil with all core Ala residues is much smaller compared with a core made up of larger bulky hydrophobes such as Leu and Ile, thus illustrating the importance of packing interactions in maintaining proper coiled-coil structures (25, 68).

Previous studies by Zhou et al. (10) have shown that Leu to Ala substitutions in a stabilizing cluster of a 35-residue homologous two-stranded coiled-coil (all 10 positions in the hydrophobic core were occupied by Leu) resulted in a destabilization of 2.9–3.0 kcal/mol for central hydrophobic core positions 9, 12, 16, 19, and 23; these values are in agreement with our results. Furthermore, Lu and Hodges (35) have recently shown that a single Leu residue with a destabilizing cluster on either side actually destabilizes the coiled-coil relative to Ala (GGGG → GGGGG). Interestingly, this situation is never observed in myosin or tropomyosin, suggesting that nature does not put Leu in such a context because it would end up destabilizing the coiled-coil further (by 0.4 kcal/mol) in an already destabilized region.

Overall our experimental results and previous studies (10, 13, 14, 35) suggest that the surrounding environment can affect the contribution of a Leu residue to stability from a low of 0.4 kcal/mol of destabilization up to a high of 3.8 kcal/mol of stabilization, depending on its context in the hydrophobic core. This type of information is critical for the understanding of the variation in regional stability throughout a coiled-coil and the contribution of each region to overall stability, function, and folding.

Acknowledgments—We gratefully thank Marc Genest and Erin Carlson for synthesis and purification of peptides. We thank Brian Tripet and Stephen Lu for their valuable discussions. CD and analytical ultracentrifugation were carried out at the Biophysics Core Facility, University of Colorado Health Sciences Center.
The Effect of a Destabilizing Cluster on Protein Stability

61. Zhou, N. E., Zhu, B.-Y., Kay, C. M. & Hodges, R. S. (1993) in Peptides: Biology and Chemistry (Proceedings of the 1992 Chinese Peptide Symposium) (Zhang, Y.-S., ed) pp. 217–220, Escom Science Publishers, Leiden, The Netherlands
62. Sonnichsen, F. D., Van Eyk, J. E., Hodges, R. S. & Sykes, B. D. (1992) Biochemistry 31, 8790–8798
63. Zhou, N. E., Kay, C. M. & Hodges, R. S. (1993) Biochemistry 32, 3178–3187
64. Karpplus, M. (1997) Protein Sci. 6, 1302–1307
65. Skolnick, J. (1983) Macromolecules 16, 1069–1083
66. Skolnick, J. & Holtzer, A. (1986) Biochemistry 25, 6192–6202
67. Goodman, E. M. & Kim, P. S. (1991) Biochemistry 30, 11615–11620
68. Holtzer, M. E., Lovett, E. G., d’Avignon, D. A. & Holtzer, A. (1997) Biophys. J. 73, 1031–1041
69. Brown, J. H., Kim, K. H., Jun, G., Greenfield, N. J., Domínguez, R., Volkmann, N., Hitchcock-DeGregori, S. E. & Cohen, C. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8496–8501
70. Singh, A. & Hitchcock-DeGregori, S. E. (2003) Biochemistry 42, 14114–14121
Stabilizing and Destabilizing Clusters in the Hydrophobic Core of Long Two-stranded α-Helical Coiled-coils
Stanley C. Kwok and Robert S. Hodges

J. Biol. Chem. 2004, 279:21576-21588.
doi: 10.1074/jbc.M401074200 originally published online March 11, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M401074200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 66 references, 14 of which can be accessed free at http://www.jbc.org/content/279/20/21576.full.html#ref-list-1