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Identification of a Unique “Stability Control Region” that Controls Protein Stability of Tropomyosin: A Two-Stranded α -Helical Coiled-Coil

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Summary

Nine recombinant chicken skeletal α -tropomyosin proteins were prepared, eight C-terminal deletion constructs and the full length protein (1–81, 1–92, 1–99, 1–105, 1–110, 1–119, 1–131, 1–260 and 1–284) and characterized by circular dichroism spectroscopy and analytical ultracentrifugation. We identified for the first time, a stability control region between residues 97 and 118. Fragments of tropomyosin lacking this region (1–81, 1–92, and 1–99) still fold into two-stranded α -helical coiled-coils but are significantly less stable (T_m between 26–28.5°C) than longer fragments containing this region (1–119, 1–131, 1–260 and 1–284) which show a large increase in their thermal midpoints (T_m between 40–43°C) for a ΔT_m of 16°C between 1–99 and 1–119. We further investigated two additional fragments which ended between residues 99 and 119, that is fragments 1–105 and 1–110. These fragments were more stable than 1–99 and less stable than 1–119 and showed that there were three separate sites that synergistically contribute to the large jump in protein stability (two electrostatic clusters, 97–104 and 112–118 and one hydrophobic interaction from Leu 110. All the residues involved in these stabilizing interactions are located outside the hydrophobic core **a** and **d** positions which have been shown to be the major contributor to coiled-coil stability. Our results clearly show that protein stability is more complex than previously thought and unique sites can synergistically control protein stability over long distances.

Keywords

protein stability; tropomyosin; two-stranded α -helical coiled-coils; stability control sites; stability control region; protein folding

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Introduction

One of the major goals of protein research is to understand the relationship between amino acid sequence and protein structure and function, since it has been observed that even a single mutation in the sequence of a protein can lead to a disease state. Intimately tied to protein structure and function is the effect of amino acid substitutions on protein folding and stability. Obviously, a threshold stability is required to initiate protein folding and therefore understanding all of the complex inter-residue interactions involved in determining protein stability is key to understanding conformational change in proteins and their function.

The ultimate goal of our research is to predict protein stability from amino acid sequence information. To reduce complexity we chose to study one of nature's most simplistic protein motifs, the two-stranded α -helical coiled-coil for the following reasons: first, it contains only one type of secondary structure, the α -helix, that is, there is no other elements of structure (β -sheet, β -turns or undefined structure) to complicate interpretation of results; second, only two α -helices are required to introduce tertiary and quaternary structure; third, the rod-like nature of the two-stranded α -helical coiled-coil simplifies the understanding of protein folding and stability to a one-dimensional problem rather than a three-dimensional problem observed in globular proteins; fourth, all of the non-covalent interactions that stabilize the three-dimensional structure of globular proteins are found in coiled-coils; fifth, it is an abundant protein motif with diverse functional roles¹; sixth, its diversity in polypeptide chain length, from the short, less than 35 residues in DNA binding domains,² to intermediate lengths, e.g. 284 residues in tropomyosin to even longer than 1,000 residues in myosin coiled-coils makes it an ideal system to test predictions of factors responsible for protein stability; and seventh, it is easy to experimentally monitor folding and stability of coiled-coils using circular dichroism spectroscopy. Thus, for the research described in this manuscript, we chose to study tropomyosin since it is the most extensively studied parallel two-stranded α -helical coiled-coil known and of sufficient length to be representative of all long coiled-coils exceeding 100-residues.

Another advantage of studying coiled-coils is that once successful in understanding protein stability of parallel two-stranded coiled-coils we have the opportunity to extend our studies to more complex coiled-coil systems because of their diversity in structure¹. There are variances in helix orientation (parallel or antiparallel), oligomerization state (two to five α -helices) and oligomerization specificity (homo- or hetero-oligomerization). This diversity in structure allows for the multiplicity in functions of native coiled-coils.

The two-stranded α -helical coiled-coil structure was first proposed by Crick (1953)³ in the absence of amino acid sequence information and prior to the determination of the three-dimensional structures of proteins by x-ray crystallography. This simple protein folding motif consists of two amphipathic, right-handed α -helices that adopt a left-handed super coil, analogous to a two-stranded rope where the non-polar face of each α -helix is continually adjacent to that of the other helix. Tropomyosin (TM) was the first α -helical coiled-coil protein to have its amino acid sequence determined^{4,5}. From this sequence we identified a hydrophobic repeat responsible for the formation and stabilization of the coiled-coil structure (N X X N X X X N X X N X X X N... where N is a non-polar residue). This repeating sequence of seven amino acid residues is denoted (**a b c d e f g**)**n** where positions **a** and **d** are occupied predominantly by hydrophobic/non-polar residues. This hydrophobic repeat was continuous and uninterrupted throughout the entire 284-residue polypeptide chain of tropomyosin. We then verified the importance of hydrophobic residues at positions **a** and **d** in the formation and stability of coiled-coils by designing and synthesizing by solid-phase peptide chemistry the first model coiled-coil protein⁶. Furthermore, we have demonstrated the effect of chain length on the stabilization and formation of two-stranded α -helical coiled-coils⁷⁻⁹. These studies

clearly demonstrated the utility of small model coiled-coil proteins for studying protein folding and stability of α -helical proteins in general.

The first high resolution X-ray structure of a parallel two-stranded α -helical coiled-coil, GCN4, a 33-residue DNA-binding protein was determined in 1991¹⁰. This structure as predicted by Crick (1953)³ showed the individual α -helices wrapping around each other to form a quarter turn of the left-handed super coil. The individual α -helices with a crossing angle of 18° were smoothly bent because the main chain hydrogen bonds at the interface were shorter than those on the outside of the helices. This difference in hydrogen bond length creating a natural curvature is a property of amphipathic α -helices¹¹. Recent inspection of the Brookhaven Protein Data Bank (PDB) has revealed 552 high resolution protein structures containing the coiled-coil motif, out of 29,509 protein entries that have been solved by X-ray crystallography and NMR. The structures clearly show that the **a** and **d** positions are almost totally buried in the coiled-coil and are structurally distinct, that is, the side-chains at these positions pack differently and have different orientations relative to the coiled-coil axis¹⁰. Analysis of coiled-coils has revealed that though hydrophobic amino acids predominate at positions **a** and **d** in the hydrophobic core of coiled-coils, all other amino acids with the exception of Pro are tolerated in these positions¹². This led Hodges and coworkers to design a model coiled-coil protein and determine the effect of substituting 20 different amino acid residues at position **a** and **d** in the center of the coiled-coil. These authors showed that single amino acid substitutions in these positions can make a wide range of contributions to stability depending on the residue (a range of stability of ~ 7 kcal/mole at each **a** and **d** position)^{13–15}. Based on this experimentally derived stability data an algorithm, STABLECOIL, was developed to predict the location of coiled-coils in protein sequences (<http://biomol.uchsc.edu/researchFacilities/ComputationalCore/stablecoil/index.html>).

To predict the overall stability of a protein one must not only identify all of the different types of non-covalent interactions contributing to protein stability but must also have a quantitative value for each interaction. In the case of the two-stranded coiled-coil, due to the rod-like nature of the coiled-coil it is reasonable to assume that the contributions to overall protein stability are additive. Numerous studies of α -helices, coiled-coils, and globular proteins have identified interactions affecting coiled-coil and protein stability in general, including polypeptide chain length^{7–9,16–18}, stability contributions from residues in the hydrophobic core (**a** and **d**) positions^{13–15} and references therein, side-chain van der Waals packing effects^{19–21}, translational and rotational entropy²², interchain and intrachain electrostatic interactions^{23–35} and references therein, side-chain helical propensities^{36–39}, helix-dipole effects⁴⁰ and references therein, N and C-terminal helix capping effects^{41–43} and references therein, control of oligomerization state^{10, 15, 44–49}, controlling parallel and antiparallel orientation of α -helices in coiled-coils^{50–53} and references therein, controlling homo-versus heterodimerization in coiled-coils^{24, 54–61} and several different types of intra-helical side-chain: side-chain interactions^{62–64}. *De novo* designed coiled-coils have been used for example, for a wide-range of applications^{54,57,65,66}.

Though there has been a massive accumulation of knowledge about the interactions that stabilize α -helices and coiled-coils, recent results suggest that there may be many more unique interactions that need to be discovered in coiled-coils before we will be able to predict protein stability from amino acid sequence, in general. First, we recently determined a 1.17Å° resolution X-ray crystal structure of a coiled-coil and identified three new stabilizing interactions: a unique hydrogen bonding-electrostatic network not previously observed in coiled-coils and two other hydrophobic interactions involving Leu residues at positions **e** and **g** from both g-a' and d-e' interchain interactions with the hydrophobic core⁶⁷. Second, an intriguing study by Kammerer *et al*, 1998⁶⁸ demonstrated that the stability of the hydrophobic core alone is not always a reliable determinant of protein folding. They proposed the idea that

a “trigger sequence” may exist within coiled-coil domains since deletion constructs of cortaxillin I and myosin II lacking a 14-residue sequence failed to fold^{69, 70}. Analysis of the trigger site revealed a distinct network of interhelical and intrahelical salt bridges⁷¹. A seven-residue trigger site in the three-stranded human macrophage scavenger receptor coiled-coil domain was identified further supporting the idea of a trigger site stabilizing the coiled-coil in order to allow the folding process to take place⁷². Our laboratory had previously addressed the question, are trigger sequences essential in the folding of two-stranded α -helical coiled-coils? We showed that a specific consensus trigger sequence is not essential for coiled-coil formation and concluded that any sequence that increases overall coiled-coil stability beyond a threshold level under given conditions allows folding to occur⁷³. Nevertheless, the ability to identify trigger sites in coiled-coils, when they exist, and quantitate their contribution to the overall stability of the folded protein is essential. Third, amino acid sequence analyses of long-coiled-coil proteins (>100 residues) that differ in composition, function and length have revealed remarkable similarity in their amino acid arrangement in the hydrophobic core. For example, the core **a** and **d** residues of tropomyosin (284 residues per polypeptide chain) and myosin heavy chain (1,086 residues) are organized into clusters (consecutive core positions) with similar hydrophobic nature, rather than having hydrophobicity randomly distributed along the entire length of the coiled-coil. We termed these, stabilizing and destabilizing clusters and defined a cluster as three or more consecutive core residues of non-polar stabilizing residues (consisting of one of six hydrophobes, Leu, Ile, Val, Met, Phe and Tyr) or three or more consecutive core residues of destabilizing residues (consisting of amino acid residues Gly, Ala, Cys, Ser, Thr, Asn, Gln, Asp, Glu, Arg, His and Lys) which are small non-polar, polar or charged side-chains. In addition, these two coiled-coils have similar numbers of stabilizing and destabilizing residues in core **a** and **d** positions, 58% and 42%, respectively⁷⁴. We have also showed that the organization of clusters in the hydrophobic core of coiled-coils creates regions of varying stabilities, thus allowing a dynamic protein molecule consisting of subdomains of high and low stability. Alternating these regions could be important in controlling the overall stability of the protein where regions of higher stability could be important in maintaining structure and regions of less stability could be important for interactions with other biomolecules or modulating overall stability/flexibility. We also demonstrated that a minimum of 3 consecutive stabilizing residues is required to form a cluster and concluded that the role of hydrophobicity in the hydrophobic core of coiled-coils is extremely context dependent and clustering is an important aspect of protein folding and stability^{75, 76}. Interestingly, in general the stabilizing clusters in tropomyosin and myosin ranged in size from 3 to 6 consecutive stabilizing residues in the hydrophobic core **a** and **d** positions and destabilizing clusters from 3 to 5 consecutive destabilizing residues⁷⁴. Both calorimetric and pressure denaturation experiments have shown independent domains with different stabilities persist along the entire length of the tropomyosin coiled-coil^{77–81}. Crystallographic evidence and mutational studies have suggested that regions of instability in tropomyosin are important to provide the necessary flexibility^{82–85} required for actin binding and that increasing the stability of the coiled-coil in the actin binding region increased overall protein stability but had an adverse effect on binding/function^{86,87}.

In our analysis of long two-stranded α -helical coiled-coils⁷⁴ we showed that in addition to stabilizing and destabilizing clusters of 3 or more stabilizing residues or 3 or more destabilizing residues in a row in the hydrophobic core **a** and **d** positions, there were also intervening regions lacking clusters which contain almost equal numbers of stabilizing and destabilizing residues. The stabilizing clusters, destabilizing clusters and intervening regions were well distributed throughout the sequences of myosin and tropomyosin⁷⁴. An example of an intervening region in tropomyosin is shown in Figure 1 where the residues in the hydrophobic core are Met127a, Ile130d, Ala134a, Asp137d, Met141a, Gln144d and Leu148a. One might postulate that intervening regions provide intermediate stability in the hydrophobic core between high stability of stabilizing clusters and low stability of destabilizing clusters. It is interesting that

a trigger site first identified in cortexillin I which was essential for coiled-coil folding^{68,69} does not involve a stabilizing cluster in the hydrophobic core (the 14-residue trigger site contains Ra, Ld, La and Td in the hydrophobic core). These results further emphasize that the hydrophobic core alone does not control the stability of coiled-coils and that other interactions outside of the hydrophobic core can dramatically enhance stability.

The question we wanted to address in this study, is whether there is a particular stabilizing cluster or nucleation domain along the sequence which triggers folding and confers the final stability of the native protein (trigger site) or whether there is a unique region that does not control folding but does control the final stability of the protein (stability control region). We will refer to the latter region as a “stability control region” to distinguish it from a “trigger site” which is essential to trigger folding. That is, C-terminal truncation sequences that contain this “stability control region” will confer the overall stability of tropomyosin and sequences that do not contain this region will fold but will have stabilities dramatically lower than that of the native protein.

Results

C-terminal truncated α -tropomyosin recombinant proteins

It has been known for more than 25 years that N-terminal region of tropomyosin is significantly more stable than the C-terminal region⁸⁸. C-terminal fragments compared to tropomyosin are less stable ($\Delta T_m > 10^\circ\text{C}$) and have been studied extensively^{88, 89} and references therein. Therefore, any “stability control region” that controls the overall stability of native tropomyosin would most likely reside in the N-terminal region. We prepared nine recombinant chicken skeletal α -tropomyosin proteins, eight C-terminal deletion constructs and the full length protein (1–81, 1–92, 1–99, 1–105, 1–110, 1–119, 1–131, 1–260 and 1–284). The lengths of the constructs were varied based on our knowledge of the location of stabilizing and destabilizing clusters and intervening regions (Figure 1).

Our recent results with model coiled-coil proteins have suggested that the contribution of stabilizing residues in the hydrophobic core positions (**a** and **d**) of the coiled-coil to protein stability is context dependent, that is, dependent on the nature of the adjacent core residues^{75, 76}. Clusters of 3 or more consecutive stabilizing hydrophobic residues in positions **a** and **d** (Leu, Ile, Val, Met, Phe and Tyr) contribute more to coiled-coil stability than when these same hydrophobic residues are separated by destabilizing residues. From the sequence of tropomyosin (Figure 1), the majority of stabilizing clusters are located near either termini, with 4 stabilizing clusters in the N-terminal region 1–99 and 5 stabilizing clusters in the C-terminal region 169–284. Therefore, we prepared fragment 1–81 which contains three stabilizing clusters and ends in a destabilizing cluster consisting of Ala74, Ala78 and Ala 81. This destabilizing cluster at the C-terminal end of the fragment is expected to be destabilizing to the overall stability of the fragment. In comparison, fragment 1–92 ends with an additional 3-residue stabilizing cluster (Val85, Leu88 and Ile92) and one might expect this fragment to be considerably more stable than fragment 1–81. Similarly, 1–99 further extends the stabilizing cluster to involve 5 consecutive stabilizing residues (Val85, Leu88, Ile92, Val95 and Leu99) in the hydrophobic core at the C-terminal of this fragment. Fragment 1–99 would be expected to be even more stable than 1–92. Fragments 1–119 and 1–131 further extend the coiled-coil sequence with an intervening region and destabilizing cluster (Figure 1). Based on the stability coefficients for the **a** and **d** positions^{14, 15} and chain length effects⁹ one would expect these two fragments to be less stable than 1–99. Thus, the predicted order of stability of these five fragments based solely on stabilizing clusters, their location and chain length effects would be 1–81 < 1–92 < 1–99 > 1–119 and 1–131. The last fragment prepared was 1–260 which deleted the region 261–284 which was suggested by Paulucci *et al*, 2002⁸⁹ to contain a region critical to the overall stability of C-terminal fragments of tropomyosin.

Stability profile of TM and calculated relative stability of the fragments

A stability profile of tropomyosin using a 14-residue window that shifts by one residue sequentially along the sequence is shown in Figure 2. This 14-residue window sums the values of α -helical propensity at positions **b**, **c**, **e**, **f** and **g**³⁷ and stability coefficients for positions **a** and **d**^{14, 15}. Since the α -helical propensity values were determined in a single-stranded α -helical model they have been proportionately adjusted to represent their relative contributions when in a coiled-coil. The profile shows that the high stability regions correspond to the location of the regions of the hydrophobic clusters of stabilizing residues and the low stability regions correspond to the location of the regions of destabilizing clusters. The residues in the hydrophobic core (**a** and **d** position) are denoted above and below the peaks and troughs in the plot (Figure 2).

Biophysical characterization of the TM constructs

Remarkably, all TM constructs, including the relatively short TM (1–81), were nearly 90% helical at 5°C in benign conditions at 3–5 μ M monomer (Figure 3, Table 1). Even the shortest fragment did not exhibit significant helix induction in the helix promoting condition of 50% trifluoroethanol (TFE). Furthermore, the molar ellipticity 222/208 ratios for all analogs is greater than 1 (ranging from 1.02 to 1.12) illustrating that coiled-coils are fully formed whereas single-stranded α -helices in 50% TFE the 222/208 ratio is less than 1^{7, 90}. Despite our original design hypothesis, the number of stabilizing clusters does not seem to have any significant effect on the overall α -helical content. We confirmed the oligomerization states of these TM fragments using sedimentation equilibrium experiments with global analyses at 3 different concentrations and 3 different speeds. In the presence of DTT and low protein concentrations (≤ 20 μ M dimer), TM fragments 1–81, 1–92 and 1–99 underwent a monomer-dimer equilibrium at speeds ranging from 8000K, 12000K and 16000K with an apparent molecular mass that is less than a dimer. Larger TM fragments TM 1–119 and beyond exhibited dimeric oligomerization states under the conditions tested (Table 1). The relative stabilities of these TM fragments were then measured by thermal denaturation. Despite similar high helical content at 5°C among the TM fragments, we observed a large jump in stability as we extended the TM fragment beyond 99 residues (Figure 4). A large thermal midpoint difference (ΔT_m 16°C between 1–99 and 1–119) distinguish the instability of the shorter TM fragments (< 100 residues) compared to those of 119 residues and longer. All the short TM constructs shared a T_m between 26–28.5°C whereas longer fragments have a native TM-like midpoint between 40–43°C. Again to our surprise, the stabilities of these fragments did not correlate with the distribution of stabilizing clusters, but rather, the region between 100–119 seemed to confer this increased stabilization. We refer to this region as a “stability control region” for tropomyosin because the presence of this region confers native like stability. One interesting feature in region 100–119 is highlighted by the heptad sequence 112–118 which is shown in Figure 5 and denoted electrostatic cluster 2. This 7-residue region contains a multitude of *i* to *i* + 3 and *i* to *i* + 4 intrachain electrostatic attractions and interchain *i* to *i*' + 5 electrostatic attractions in each chain of the two-stranded α -helical coiled-coil. Despite having only one stabilizing Leu as a core residue, this single heptad has four electrostatic attractions or a total of 8 electrostatic attractions in the two-stranded coiled-coil. Thus, we postulate that this unique heptad sequence KLEEA EK must provide electrostatic interactions that contribute substantially more to stability than individual electrostatics previously identified in coiled-coils. The contributions of *i* to *i*' + 5 (g-e') salt bridges in coiled-coils to stability, range on average between 0.4 to 0.6 kcal/mol per salt bridge^{25, 35, 91}. It has also been shown that orientation of the salt bridge favors the Kg-Ee' over the Eg-Ke' orientation^{23, 35, 92}. Orientation and spacing for salt-bridges in α -helices has been studied⁹³. In addition, it has been demonstrated that when there are multiple electrostatic attractions possible (interchain and intrachain) between the same charged residues or a network of interactions there is a large enhancement to stability over the sum of individual interactions^{67, 71, 92, 94}. This leads us to

hypothesize that this site, because of its large number of intra- and interchain electrostatic attractions, must contribute significantly more to stability relative to any other individual heptad in tropomyosin.

Another interesting feature in region 100–119 is highlighted in the 8-residue sequence 97–104 which is shown in Figure 5. This 8-residue region contains a multitude of i to $i + 3$ and i to $i + 4$ intrachain electrostatic attractions. Despite having only one stabilizing Leu as a core residue, this 8-residue sequence has three electrostatic attractions in each chain of the two-stranded α -helical coiled-coil. Thus, we postulate that this sequence EELDRAQE could also provide electrostatic interactions that contribute substantially more to stability than individual electrostatics previously identified in coiled-coils.

Recently, a statistical analysis of intrahelical ionic interactions in parallel coiled-coils was carried out¹⁰². Their results suggest some general conclusions: first, that ionic interactions containing glutamic acid contribute more to coiled-coil stability and formation than those involving aspartic acid. The two electrostatic clusters 1 and 2 identified in this study contain only glutamic acid involved in i to $i + 3$ and i to $i + 4$ intrachain electrostatic attractions (Fig. 5); second, the ion pairs with high probability of formation and high frequency of occurrence (3 ER and 4 ER) are found in cluster 1; third, ion pairs with medium probability of formation and an intermediate frequency of occurrence (3 RE, 3 KE, 4 EK and 3 EK) are found in cluster 1 and 2.

Uniqueness of the heptad sequence KLEEA EK (residues 112–118)

To determine the uniqueness of this seven residue sequence, KLEEA EK, we created a data base of all tropomyosin sequences (see Materials and Methods section). This data base consisted of 379 different tropomyosin sequences from different species and different isoforms in a given species. We then created a heptad data base where all the heptads started at position **g** and ended at position **f** (**g a b c d e f**). Positions **g** and **e** indicate if a g to e' interchain salt bridge exists in the coiled-coil. Positions **a** and **d** are the hydrophobic core residues responsible in a major way to the formation and stability of coiled-coils. The heptad data base consisted of 13,647 heptads from the 379 different tropomyosin sequences. Interestingly, there are 2,144 unique heptad sequences despite the high conservation of TM sequences. In Figure 6 we plotted the occurrence of all 2,144 heptads. Ten heptad sequences have an occurrence between 208 times to 294 times suggesting that these heptads are important for structure, function and stability of tropomyosin. These ten heptad sequences are shown in Table 2 (top panel). The sequence of interest, KLEEA EK, occurs 248 times in the data base (the third most frequent occurring heptad) and has the greatest number of possible electrostatic attractions of these ten heptads (2 intrachain, i to $i + 3$; 1 intrachain i to $i + 4$ and 1 interchain i to $i' + 5$ for a total of four) (Table 2).

If we search for heptads that contain an interchain i to $i' + 5$ salt bridge we find 4,594 heptads or 34% of all heptads in tropomyosin contain an interchain electrostatic attraction to stabilize the coiled-coil. In contrast, if we search for heptads that have simultaneously an interchain and both intrachain i to $i + 3$ and i to $i + 4$ salt bridges we find only 294 heptads or 2% of the 13,647 heptads contain this kind of heptad. In addition, these heptads are never found more than once in any tropomyosin sequence. Of the 294 heptads 248 heptads have the sequence KLEEA EK or 84% and are located in the same region of tropomyosin represented by the 35-residue sequence RIQLVEEELDRAQERLATALQKLEEA EKAADESER in region 91–125 (Fig. 1). This region contains five of the top ten highest occurring heptads in tropomyosin (Region 1, Table 2 and 3), including the potential stability control sites, 98–104 and 112–118 (Fig. 6, Table 2). Of the remaining 46 heptads, though homologous to the KLEEA EK sequence (Table 3) 40 heptads are never found in this region of tropomyosin. In fact, they are located more to the N-terminal end of tropomyosin represented by the 42-residue sequence

KMQQVENELDQVQEQLSLANT**KLEEKEK**ALQNAEGEVAALNR (region 49–90; Region 2, Table 3). It suggests that all homologs of the stability control site KLEEA EK may provide the same role in other tropomyosins. At position **g** and **f** lysine was 100% conserved. At position **b**, **c**, and **e** the occurrence of glutamic acid was 97.6%, 99.7% and 93.9% with aspartic acid being the only replacement (Table 3). In the hydrophobic core positions Leu is highly conserved 293/294 or 99.7% at position **a** and Ala is highly conserved at position **d**, Ala (85.0%), Lys (9.9%), Thr (5.1%) and Leu (0.7%) (Table 3). These results clearly show the uniqueness of this heptad sequence in tropomyosins supporting its role as a stability control site.

We used another approach to evaluate the uniqueness of the KLEEA EK sequence which was to determine the number of times a sequence of the same composition but different sequence appears. In this search we maintained the hydrophobic core residues at positions **a** and **d** (Leu and Ala, respectively). As shown in Table 2 (bottom) there are 9 such sequences. Sequences 1–3 and sequences 5 and 6 in Table 2 have interchain *i* to *i'* + 5 electrostatic attractions and intrachain electrostatic attractions while sequences 4 and 7–9 have intrachain electrostatic attractions only. Interestingly, 8 of the 9 sequences of same composition but different sequence were not observed in tropomyosin and one sequence was found only once in tropomyosin (Table 2). These 9 sequences were found in other proteins in the sequence data base 754 times and only once in tropomyosin. These results also support the unique properties of the KLEEA EK sequence.

The sequence with the highest homology to the KLEEA EK sequence is the sequence KLEEKEK where Ala at position **d** contains a Lys residue. This heptad sequence occurs at a different location in tropomyosin than the KLEEA EK sequence (Table 3). Nevertheless, the KLEEKEK sequence may provide a similar role in these other tropomyosins. Lys at position **d** has been shown to be considerably more destabilizing than Ala at this position in model coiled-coils¹⁵ and *i* to *i* + 3 or *i* to *i* + 4 intrachain ionic attractions do not exist with Lys at position **d** in this sequence. Thus, it is difficult to see any role for Lys at position **d** other than a destabilizing role relative to Ala at this position. Lys could be modulating the overall contribution of this stability control site.

Fragments ending within the stability control region 100–119

Two additional chain-length fragments were prepared, 1–105 and 1–110, that ended in the stability control region between 99 and 119. Based only on the stability clusters and their location, these fragments would not be expected to increase stability compared to fragment 1–99. As shown in Fig. 5, extension of the sequence from 1–99 to 1–105 adds an Ala in the hydrophobic core at position 102d which contributes very little to stability based on the stability coefficients of residues in the hydrophobic core of coiled-coils.¹⁵ On the other hand, this extension adds R101 which adds a potential of three intra-chain electrostatic interactions in the eight residue sequence, EELDRAQE, in each chain of the two-stranded coiled-coil (Fig. 5). We refer to this 8-residue region as electrostatic cluster I (97–104). For monomer concentrations of 35–52 μ M, the T_m of fragment 1–105 was 33°C, compared to fragment 1–99 with a T_m of 29°C. This increase in stability (ΔT_m of 4°C) can only be explained by the contribution of electrostatic cluster I to the stability of the coiled-coil (Fig. 5 and Fig. 7A).

Fragment 1–110 contains electrostatic cluster I (97–104) discussed above and extends the polypeptide chain to residue 110. This sequence was chosen to exclude electrostatic cluster II, 112–118 (KLEEA EK). Thus, we could compare the stability of fragment 1–110 with fragment 1–105 which contains electrostatic cluster I and fragment 1–119 which contains both electrostatic clusters I and II (Fig. 5 and Fig. 7A). Our expectation was that fragment 1–110 would show no increased stability since the extension contains an Ala residue at position 109 which is known to be very destabilizing relative to Leu in the hydrophobic core¹⁵ and no

additional electrostatics were added compared to 1–105. To our surprise, the T_m of fragment 1–110 was 37°C. This increase (ΔT_m of 4°C) can only be explained by a significant contribution of Leu at position 110e to stability. It has been previously shown by our laboratory that large hydrophobes like Leu at positions **e** and **g** can contribute to stability even though they are not in the hydrophobic core.⁶⁷ However, this increase in stability was not anticipated because of the Ala residue at position 109d (Fig. 5) in the hydrophobic core. The stability of the fragments 1–99 (T_m 29°C), 1–105 (T_m 33°C), 1–110 (T_m 37°C) and the 1–119 (T_m 47°C) shows that electrostatic cluster II (112–118) contributes a ΔT_m of 10°C to stability (Fig. 7). Thus, the dramatic jump in stability observed between 1–99 and 1–119 (ΔT_m of 18°C) involves three sites: electrostatic cluster I, 97–104; electrostatic cluster II, 112–118 and the hydrophobic contribution of Leu 110. Together these three sites account for a ΔT_m of 16–18°C between fragments 1–99 and 1–119 over a monomeric concentration range of 3–52 μ M (Table 1 and Fig. 7A).

In order to explore the effect of concentration on the stability of these coiled-coils and to fully understand the nature of their thermal unfolding, we measured the thermal stability of TM 1–119 in a series of different concentrations. Panel B, Fig. 7 shows the temperature denaturation of TM 1–119 at 52 μ M, 16 μ M, 3.1 μ M, 0.63 μ M and 0.31 μ M of monomer. This coiled-coil follows a cooperative unfolding with increasing temperature over a 165 fold concentration range. The T_m increases with increasing peptide concentration as expected as the monomer-dimer equilibrium shifts to increasing dimer formation with increasing protein concentration.

Discussion

We have shown previously that a coiled-coil with Ala relative to Leu at position **d** destabilized the coiled-coil by 3.8 kcal/mol¹⁵ yet this “stability control region” adds dramatic stability to tropomyosin even with three Ala residues at positions 102d, 109d and 116d (Fig. 5). It has been shown that Leu at position **d** not only provides the highest stability to model coiled-coils, it has the highest occurrence at this position in native coiled-coils. This result suggests that the smaller Ala residue in the hydrophobic core may be a requirement to allow more favorable electrostatic attractions in this region and a more favorable hydrophobic packing from Leu 110. These interactions not only have to overcome the loss of stability by the presence of the three Ala residues instead of Leu residues in the hydrophobic core but substantially enhance stability. To add support for this observation we searched our heptad coiled-coil data base for the sequence KLEELEK with the Ala at position **d** replaced by Leu. As expected, this sequence was only observed twice in tropomyosins (0.7%). We also searched the data base for the sequence KXEEAEK to see if any other large hydrophobe (Ile, Val, Met, Phe, Tyr) other than Leu was acceptable in position **a** of this “stability control site”. As expected, only one such sequence was found in the heptad coiled-coil data base (Table 3) again suggesting the uniqueness of this site. Taken together these computational results suggest that nature has chosen a unique combination of special sites to create a region that can dramatically enhance stability.

Our results are supported by previous studies where different fragments of rabbit skeletal α -tropomyosin (highly homologous to chicken skeletal α -tropomyosin) show similar results. Constructs with this region (1–133, 13–125, 11–127, 1–169, 1–189 and 1–284) have T_m values ranging from 44–50°C and constructs lacking this region (134–284, 183–284, 183–244, 142–281, 190–284 and 170–284) have T_m values ranging from 24–37°C⁸⁸. All the T_m values for this study and that of Pato *et al*, 1981⁸⁸ were carried out in 100 mM KCl, 50 mM PO₄ buffer, pH 7 containing DTT to prevent disulfide bridge formation at cysteine 190 in a hydrophobic core position and are directly comparable.

Further support for our discovery of a stability control region is the result from the deletion mutant of rat striated muscle α -tropomyosin (highly homologous to rabbit and chicken), 1–284 del 89–123. This construct deletes our “stability control region” identified in this study. The deletion mutant had a stability decrease of $\sim 10^{\circ}\text{C}$ compared to wild-type tropomyosin⁹⁵. The conditions for determining T_m values was different (10 mM sodium phosphate buffer, pH 7.5 containing 500 mM NaCl). High salt (500 mM) is known to significantly stabilize tropomyosin by 10°C in T_m compared to its absence⁹⁶. Nevertheless, their study does support the need for the “stability control region” to achieve native protein stability.

Paulucci *et al* (2002)⁸⁹ suggested that region 261–284 was important for the stability of the C-terminal half of tropomyosin (fragment 143–260 and 143–284 had $[\text{urea}]_{1/2}$ values of 1.35 M and 2.3 M, respectively). Fragment 183–244 was significantly less stable than 183–284 (T_m values of 24° and 33°C , respectively)⁸⁸. However, this result could be explained by deletion of the hydrophobic cluster 246–260 and not the loss of 261–284. In contrast, our constructs 1–260 and 1–284 have very similar stabilities (T_m values of 40 and 43°C , respectively) suggesting that deletion of 261–284 is not that important if the constructs contain the “stability control region,” 97–118. Kammerer *et al* (1998)⁶⁸ proposed a trigger consensus sequence that is responsible for the initiation of folding in coiled-coils including a site in tropomyosin residues 226–238. The thirteen residue consensus sequence proposed by Kammerer *et al* (1998)⁶⁸ was xxLE Ex hcxcxcx where c is a charged residue, h is a hydrophobe and x is any residue. In chicken skeletal α -tropomyosin this consensus sequence clearly does not exist where the defined E of the consensus sequence has been replaced by T229 and the charged residue by T237 (226–238, KVLTDKLKEAETR). In addition, our data clearly show that this region has no affect on folding or stability of chicken skeletal α -tropomyosin since fragments 1–119 and 1–131 which do not have this region (226–238) are folded and have native TM stability (T_m 42.0 and 43.0°C , respectively) similar to fragments 1–260 and 1–284 (T_m 40.0 and 43.0°C , respectively) (Table 1).

Elucidating and identifying this “stability control region 97–118” in tropomyosin which consists of two electrostatic clusters and a special hydrophobic interaction reveals the intricacies of the types of interactions that control protein stability. The question remains is this phenomena of a “stability control region” and “stability control sites” unique to tropomyosin or are they more general and will be found in other coiled-coil proteins. This result clearly shows that stability is more than just a sum of local hydrophobic/non-hydrophobic interactions but unique sites can synergistically control protein stability over long distances.

Materials and Methods

DNA Construction

As DNA template for construction of truncated forms of chicken skeletal muscle α TM, the previously described pET3aTM expression vector was used as template^{97,101}. Each primer included a BamHI recognition sequence permitting insertion of each construct at the BamHI site of the pET3a vector. This site is immediately downstream of the T7 tag nucleotide sequence of the pET3a vector. Insertion of each construct at the BamHI site of the vector thus leads to protein translation of a fusion T7 tagged TM product consisting of N-terminal 14 residue T7 tag followed by the N-terminal sequence of TM. This was initially useful for purification of the expressed TM constructs by chromatography using a T7 peptide affinity column. Subsequently and during the progress of this study however, a one-step reversed-phase HPLC procedure was developed and adapted in this laboratory for this purpose⁹⁸.

The PCR primer encoding the N-terminal end of TM contained a 6 nucleotide non-annealing sequence (TCACGC) followed by the BamHI sequence (GGATCC) followed by codons for amino acid residues 1 to 5 of TM. To generate C-terminal truncated TMs 1–81, 1–92, 1–99,

1–260 and full length TM 1–284, the primer sequences each contained a 5 nucleotide non-annealing sequence (GTCAT) followed by the BamH1 site and two step anticodons followed by anticodons for residues 81–77 plus T, 92–88 plus G, 99–94, 260–255 and 284–278, respectively. The non-annealing sequence was included to foster efficient cleavage by the BamH1 restriction endonuclease. PCR was performed using Pfu turbo polymerase (Stratagene, La Jolla, CA) for 30 cycles at 94°C for 30 s, at 48–55°C for 30 s. (optimum annealing temperature determined empirically for each primer) and 72°C for 2 min. Each PCR generated product was digested with BamH1, dephosphorylated and ligated into the BamH1 site of pET3a vector. Correct orientation of the inserts was established and the complete nucleotide sequence of each construct confirmed by DNA sequencing.

For construction of truncated fusion TMs 1–119 and 1–131, two stop codons at residue positions 120–121 and 132–133 were introduced by site directed mutagenesis into either the 1–260 construct or full length 1–284 construct.

Protein expression and purification

Over expression of each of the recombinant proteins was performed by growth in L-broth with ampicillin selection at 37°C, in *E. coli* bacterial cells; BL21 DE3, with induction by 0.4 mM IPTG (isopropylthio- β -galactoside)¹⁰¹. Following centrifugation, the cell pellet was resuspended in 20 mM Tris-HCl, pH 7.5, for affinity column purification or with 0.1% TFA for our one-step purification on reversed-phase HPLC. The resuspended cells were sonicated and centrifuged at 10,000 $\times$ g for 10 minutes. Analysis of the cleared crude lysates on 15% SDS-polyacrylamide gel electrophoresis (PAGE) stained with coomassie brilliant blue reveals an over-expressed product in the soluble fraction. In fact, all of these different truncated fusion proteins and the full-length α -tropomyosin fusion protein were found in the soluble fraction. Confirmation of each of the truncated proteins was performed by Western Blot analysis with detection by antibody specific for the T7 Tag Lumiblot Kit (Novagen, Madison, WI). Initial purification on a T7 Tag antibody column (Novagen, Madison, WI) aided in defining the recombinant proteins, however, one-step purification on reversed-phase HPLC has subsequently been employed for all large scale preparations.⁹⁸

Protein Purification by One-Step Reversed-phase HPLC

Initial purification on a T7 Tag antibody column aided in defining the recombinant proteins, however, a one-step purification on reversed-phase HPLC was employed for all large scale preparations⁹⁸. Crude proteins were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Zorbax SB-300 C8 column (150 $\times$ 2.1 mm inner diameter, 5 μ m particle size, 300-Å pore size) by a one-step protocol. The flow rate was 300 μ l/min. and the detection wavelengths used were 210 nm and 280 nm. Briefly, the purification protocol involved three steps: first, a rapidly-rising linear AB gradient (increasing 2.0% B per min), (where eluent A was 0.05% aqueous trifluoroacetic acid and eluent B was 0.05% trifluoroacetic acid in acetonitrile) reached a concentration of acetonitrile at 15% below that of the elution concentration for the protein of interest in the crude sample (first observed on a smaller-scale analytical RP-HPLC run); second, a slower linear AB gradient (0.1–0.2% B per min, depending on lysate complexity) with the above eluents was used to separate the protein of interest; and third, an isocratic run at a high acetonitrile concentration to wash off any remaining hydrophobic impurities (e.g., 70% acetonitrile). The fractions containing the protein product were pooled, lyophilized and verified by quantitative amino acid analysis (Beckman model 6300 amino acid analyzer, Beckman-Coulter, Palo Alto, CA) and by electrospray mass spectrometry on a Mariner Biospectrometry work station (Applied Biosystems, Foster City, CA).

Analytical ultracentrifugation equilibrium experiments

Sedimentation equilibrium analysis was performed on a Beckman XLA analytical ultracentrifuge with absorbance optics at 215–225 nm for the detection of the peptide backbone. Samples were first dialyzed overnight against an aqueous solution of 100 mM KCl, 50 mM PO₄, pH 7.0 (benign buffer) containing 2 mM DTT at 4 °C. A 110-μl aliquot was loaded into the 12-mm Epon cell (charcoal-filled), and three different initial dilutions of each TM fragment stock were loaded: cell 1, 7.1–10.3 μM; cell 2, 14.2–20.6 μM and cell 3, 28.4–41.3 μM (monomer). The samples were spun at 20 °C at 12,000, 16,000, 20,000, and 24,000 rpm for at least 12 h to achieve equilibrium, as demonstrated by successive identical radial scans.

The behavior of the TM fragment species at equilibrium is described by the following equation,

$$M_{\text{buoy}} = M_m(1 - v\rho)$$

where M_{buoy} is the measured buoyant weight, M_m is the molecular mass in daltons, v is the partial specific volume of the sample, and ρ is the density of the buffer solution. The partial specific volume of the sample and density of the buffer were calculated using SednTerp (version 1.06, University of New Hampshire) using the weighted average of the amino acid composition. The TM fragment 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 ORIGINS 6.0 (version 6.0, Microcal) with XL-A/XL-I data analysis software (Beckman Coulter, Palo Alto, CA).

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 combined with Peltier control, where rectangular cells of 1 mm path length and circular cells of 0.5-mm path length were used. The concentrations of TM fragment solutions were determined by amino acid analyses. For wavelength scan analysis, a concentrated stock solution (>20 μM) of each TM fragment in 100 mM KCl, 50 mM PO₄, pH 7.0 (benign buffer) containing 2 mM DTT was diluted and scanned in the presence and absence of 50% trifluoroethanol (TFE). Mean residue molar ellipticity was calculated using the equation,

$$[\theta] = \theta_{\text{obs}} \cdot \text{mrw} / 10lc$$

where θ_{obs} 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 c is the protein concentration (mg/ml). Each protein spectrum was the average of six wavelengths scans collected at 0.1 nm intervals from 195 to 250 nm. The uncertainty in the molar ellipticity values was ± 300 degrees·cm²·dmol⁻¹. Protein stability measurements were monitored at wavelength 222 nm, indicative of the secondary structure of α -helices, by thermal denaturation. For thermal melting experiments, data points were taken at 1° C intervals at a scan rate of 60° C/hr.

Computational Analysis of Tropomyosin Sequences

The tropomyosin database was constructed as follows. A primary search for tropomyosins was conducted using the NCBI BLAST WEB interface (<http://www.ncbi.nlm.nih.gov/BLAST/>) with the nr (non-redundant) database and all other parameters defaulted^{99, 100}. The search sequence was human alpha tropomyosin 1 (gi|13938565|gbAAH07433.1). 5000 sequences

were retained from this search. From this list, all sequences whose tag contained the word tropomyosin (and its mis-spellings) or the word tpm were extracted (using a case-insensitive search). This first list was manually curated to determine the start residue of the heptad repeats. At the same time, sequences which did not fit the heptad repeat pattern or had a high content of proline residues were removed from the list. This created an initial database of 422 tropomyosin proteins. This database was analysed using the heptad start residue and assuming heptad repeats with no insertions/deletions to find all unique heptad sequences. The heptad analysis for a sequence was terminated if a heptad with 2 prolines was seen or two prolines had been seen in the sequence. The number of occurrences of each unique sequence heptad were counted. From this list and sorting by number of occurrences it was seen that the 5 most occurring unique sequence heptads actually formed a contiguous sequence of 35 residues, RIQLVEEELDRAQERLATALQKLEEAKEKADESER. This sequence was used in an ungapped BLAST search of the current tropomyosin database for specific sequences (word count 2, PAM30 matrix and gap parameters 32767 and 32767) and only sequences which had a primary ungapped match of 25 or more residues were retained to form the final tropomyosin database. This database contained 379 sequences and 13647 heptads, of which there were 2144 unique sequence heptads.

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References

1. Wolf E, Kim PS, Berger B. MultiCoil: A program for predicting two- and three-stranded coiled coils. *Protein Sci* 1997;6:1179–1189. [PubMed: 9194178]
2. Alber T. Structure of the leucine zipper. *Curr Opin Genet Dev* 1992;2:205–210. [PubMed: 1638114]
3. Crick FCH. The packing of α -helices: simple coiled-coils. *Acta Crystallog* 1953;6:689–697.
4. Hodges RS, Sodek J, Smillie LB, Jurasek L. Tropomyosin: amino acid sequence and coiled-coil structure. *Cold Spring Harbor Symp Quant Biol* 1972;37:299–310.
5. Sodek J, Hodges RS, Smillie LB, Jurasek L. Amino acid sequence of rabbit skeletal tropomyosin and its coiled-coil structure. *Proc Natl Acad Sci U S A* 1972;69:3800–3804. [PubMed: 4509342]
6. Hodges RS, Saund AK, Chong PC, St-Pierre SA, Reid RE. Synthetic model for two-stranded α -helical coiled-coils. Design, synthesis and characterization of an 86-residue analog of tropomyosin. *J Biol Chem* 1981;256:1214–1224. [PubMed: 7451500]
7. Lau SY, Taneja AK, Hodges RS. Synthesis of a model protein of defined secondary and quaternary structure: Effect of chain length on the stabilization and formation of two-stranded α -helical coiled-coils. *J Biol Chem* 1984a;259:13253–13261. [PubMed: 6490655]
8. Su JY, Hodges RS, Kay CM. Effect of chain length on the formation and stability of synthetic α -helical coiled-coils. *Biochemistry* 1994;33:15501–15510. [PubMed: 7803412]
9. Kwok SC, Hodges RS. Effect of chain length on coiled-coil stability: decreasing stability with increasing chain length. *Biopolymers* 2004a;76:378–390. [PubMed: 15372485]
10. O'Shea EK, Klemm JD, Kim PS, Alber T. X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled-coil. *Science* 1991;254:539–544. [PubMed: 1948029]
11. Zhou NE, Zhu BY, Sykes BD, Hodges RS. The relationship between amide proton chemical shifts and hydrogen bonding in amphipathic α -helical peptides. *J Am Chem Soc* 1992;114:4320–4326.
12. Lupas A, Van Dyke M, Stock J. Predicting coiled coils from protein sequences. *Science* 1991;252:1162–1164.

13. Wagschal K, Tripet B, Hodges RS. *De novo* design of a model peptide sequence to examine the effects of single amino acid substitutions in the hydrophobic core on both stability and oligomerization state of coiled-coils. *J Mol Biol* 1999a;285:785–803. [PubMed: 9878444]
14. Wagschal K, Tripet B, Lavigne P, Mant CT, Hodges RS. The role of position a in determining the stability and oligomerization state of alpha-helical coiled coils: 20 amino acid stability coefficients in the hydrophobic core of proteins. *Protein Sci* 1999b;8:2312–2329. [PubMed: 10595534]
15. Tripet B, Wagschal K, Lavigne P, Mant CT, Hodges RS. Effects of side-chain characteristics on stability and oligomerization state of a *de novo*-designed model coiled-coil: 20 amino acid substitutions in position “d”. *J Mol Biol* 2000;300:377–402. [PubMed: 10873472]
16. Lumb KJ, Carr CM, Kim PS. Subdomain folding of the coiled coil leucine zipper from the bZIP transcriptional activator GCN4. *Biochemistry* 1994;14:7361–7367. [PubMed: 8003501]
17. Fairman R, Chao HG, Mueller L, Lavoie TB, Shen L, Novotny J, Matsueda GR. Characterization of a new four-chain coiled-coil: influence of chain length on stability. *Protein Sci* 1995;4:1457–1469. [PubMed: 8520471]
18. Litowski JR, Hodges RS. Designing heterodimeric two-stranded α -helical coiled-coils: The effect of chain length on protein folding, stability and specificity. *J Pept Res* 2001;58:477–492. [PubMed: 12005418]
19. Eriksson AE, Baase WA, Zhang XJ, Heing DW, Blader M, Baldwin EP, Matthews BW. Response of a protein structure to cavity-creating mutations and its relation to the hydrophobic effect. *Science* 1992;255:178–183. [PubMed: 1553543]
20. Zhu BY, Zhou NE, Kay CM, Hodges RS. Packing and hydrophobicity effects on protein folding and stability. Effects of β -branched amino acids, valine and isoleucine, on the formation and stability of two-stranded α -helical coiled-coils/leucine zippers. *Protein Science* 1993;2:383–394. [PubMed: 8453376]
21. Yu BY, Lavigne P, Privalov PL, Hodges RS. A measure of disorder in a folded protein and its contribution to stability. *J Am Chem Soc* 1999a;121:8443–8449.
22. Yu Y, Lavigne P, Kay CM, Hodges RS, Privalov PL. Contribution of translational and rotational entropy to the unfolding of a dimeric coiled-coil. *J Physical Chemistry B* 1999b;103:2270–2278.
23. Krylov D, Mikhail I, Vinson C. A thermodynamic scale for leucine zipper stability and dimerization specificity: e and g interhelical interactions. *Emboj* 1994;13:2849–2861.
24. Zhou NE, Kay CM, Hodges RS. The role of interhelical ionic interactions in controlling protein folding and stability: De novo designed synthetic two-stranded α -helical coiled-coils. *J Molecular Biology* 1994a;237:500–512.
25. Zhou NE, Kay CM, Hodges RS. The net energetic contribution of interhelical electrostatic attractions to coiled-coil stability. *Protein Eng* 1994b;7:1365–1372. [PubMed: 7700868]
26. Monera OD, Kay CM, Hodges RS. Protein denaturation with guanidine hydrochloride or urea provides a different estimate of stability depending on the contributions of electrostatic interactions. *Protein Science* 1994a;3:1984–1991. [PubMed: 7703845]
27. Kohn WD, Kay CM, Hodges RS. Protein destabilization by electrostatic repulsions in the two-stranded α -helical coiled-coil/leucine zipper. *Protein Science* 1995a;4:237–250. [PubMed: 7757012]
28. Kohn WD, Monera OD, Kay CM, Hodges RS. The effects of interhelical electrostatic repulsions between glutamic acid residues in controlling the dimerization and stability of two-stranded α -helical coiled-coils. *J Biol Chem* 1995b;270:25495–25506. [PubMed: 7592719]
29. Yu Y, Monera OD, Hodges RS, Privalov PL. Ion-pairs significantly stabilize coiled-coils in the absence of electrolyte. *J Mol Biol* 1996a;255:367–372. [PubMed: 8568882]
30. Yu Y, Monera OD, Hodges RS, Privalov PL. Investigation of electrostatic interactions in two-stranded coiled-coils through residue shuffling. *Biophysical Chemistry* 1996b;59:299–314. [PubMed: 8672718]
31. Lumb KJ, Kim PS. Measurement of interhelical electrostatic interactions in the GCN4 leucine zipper. *Science* 1995;268:436–439. [PubMed: 7716550]
32. Lumb KJ, Kim PS. Interhelical salt-bridges, coiled-coil stability and specificity of dimerization. *Science* 1996;271:1137–1138. [PubMed: 17792302]
33. Lavigne P, Sönnichsen FD, Kay CM, Hodges RS. Interhelical salt-bridges, coiled-coil stability and specificity of dimerization. *Science* 1996;271:1136–1138. [PubMed: 8599093]

34. Kohn WD, Kay CM, Hodges RS. Salt effects on the protein stability: Two-stranded α -helical coiled-coils containing inter- or intrahelical ion-pairs. *J Mol Biol* 1997a;267:1039–1052. [PubMed: 9135129]
35. Kohn WD, Kay CM, Hodges RS. Orientation, positional, additivity, and oligomerization state effects of interhelical ion-pairs in α -helical coiled coils. *J Mol Biol* 1998;283:993–1012. [PubMed: 9799639]
36. O’Neal KT, DeGrado WF. A thermodynamic scale for the helix-forming tendencies of the commonly occurring amino acids. *Science* 1990;250:646–651. [PubMed: 2237415]
37. Zhou NE, Monera OD, Kay CM, Hodges RS. α -Helical propensities of amino acids in the hydrophobic face of an amphipathic α -helix. *Protein and Peptide Letters* 1994c;1:114–119.
38. Chakrabartty A, Kortemme T, Baldwin RL. Helix propensities of the amino acids measured in alanine-based peptides without helix-stabilizing side-chain interactions. *Protein Sci* 1994;3:843–852. [PubMed: 8061613]
39. Monera OD, Sereda TJ, Zhou NE, Kay CM, Hodges RS. Relationship of side-chain hydrophobicity and α -helical propensity on the stability of the single-stranded amphipathic α -helix. *J of Peptide Science* 1995;1:319–329.
40. Kohn WD, Kay CM, Hodges RS. Positional dependence of the effects of negatively charged Glu side-chains on the stability of two-stranded α -helical coiled-coils. *J Peptide Sci* 1997b;3:209–223. [PubMed: 9230486]
41. Aurora R, Rose GD. Helix capping. *Protein Sci* 1998;7:21–38. [PubMed: 9514257]
42. Lu M, Shu W, Ji H, Spek E, Wang L, Kallenbach NR. Helix capping in the GCN4 leucine zipper. *J Mol Biol* 1999;288:743–752. [PubMed: 10329176]
43. Tripet B, Hodges RS. Helix capping interactions stabilize the N-terminus of the kinesin neck coiled-coil. *J Struct Biol* 2002;137:220–235. [PubMed: 12064948]
44. O’Shea EK, Lumb KJ, Kim PS. Peptide “Velcro”: design of a heterodimeric coiled coil. *Curr Biol* 1993;3:658–667. [PubMed: 15335856]
45. Harbury PB, Zhang T, Kim PS, Alber T. A switch between two-, three- and four-stranded coiled coils in GCN4 leucine zipper mutants. *Science* 1993;262:1401–1407. [PubMed: 8248779]
46. Gonzalez L Jr, Brown RA, Richardson D, Alber T. Crystal structures of a single coiled-coil peptide in two oligomeric states reveal the basis for structural polymorphism. *Nature Struct Biol* 1996a;3:1002–1009. [PubMed: 8946853]
47. Gonzalez L Jr, Plecs JJ, Alber T. An engineered allosteric switch in leucine-zipper oligomerization. *Nature Struct Biol* 1996b;3:510–515.
48. Gonzalez L Jr, Woolfson DN, Alber T. Buried polar residues and structural specificity in the GCN4 leucine zipper. *Nature Struct Biol* 1996c;3:1011–1018. [PubMed: 8946854]
49. Monera OD, Sonnichsen FD, Hicks L, Kay CM, Hodges RS. The relative positions of alanine residues in the hydrophobic core control the formation of two-stranded or four-stranded α -helical coiled-coils. *Protein Engineering* 1996a;9:353–363. [PubMed: 8738211]
50. Monera OD, Zhou NE, Kay CM, Hodges RS. Comparison of antiparallel and parallel two-stranded α -helical coiled-coils: Design, synthesis and characterization. *J Biol Chem* 1993;268:19218–19227. [PubMed: 8366074]
51. Monera OD, Kay CM, Hodges RS. Electrostatic interactions control the parallel and antiparallel orientation of α -helical chains in two-stranded α -helical coiled-coils. *Biochemistry* 1994b;33:3862–3871. [PubMed: 8142389]
52. Monera OD, Zhou NE, Lavigne P, Kay CM, Hodges RS. Formation of parallel and antiparallel coiled-coils controlled by the relative positions of alanine residues in the hydrophobic core. *J Biol Chem* 1996b;271:3995–4001. [PubMed: 8626731]
53. Zoetewey DL, Tripet B, Kutateladze T, Overduin M, Wood JM, Hodges RS. NMR solution structure of the cytoplasmic antiparallel coiled-coil domain from *Escherichia coli* ProP. *J Mol Biol* 2003;334:1063–1076. [PubMed: 14643666]
54. Tripet B, Yu L, Bautista D, Wong WY, Irvin RT, Hodges RS. Engineering a *de novo* designed coiled-coil dimerization domain for rapid detection, purification and characterization of recombinant peptides and proteins. *Protein Engineering* 1996;9:1029–1042. [PubMed: 8961356]

55. Lavigne P, Crump MP, Gagne SM, Hodges RS, Kay CM, Sykes BD. Insights into the Mechanism of heterodimerization from the ¹H-NMR solution structure of the c-MYC-MAX heterodimeric leucine zipper. *J Mol Biol* 1998;281:165–181. [PubMed: 9680483]
56. Chao H, Houston M, Grothe S, Kay CM, O'Connor-McCourt M, Irvin RT, Hodges RS. Kinetic study on the formation of a *de novo* designed heterodimeric coiled-coil: Use of surface plasmon resonance to monitor the association and dissociation of polypeptide chains. *Biochemistry* 1996;35:12175–12185. [PubMed: 8810925]
57. Chao H, Bautista DL, Litowski J, Irvin RT, Hodges RS. The use of a heterodimeric coiled-coil system for biosensor application and affinity purification. *J Chromatography B* 1998;715:307–329.
58. Litowski JR, Hodges RS. Designing heterodimeric two-stranded α -helical coiled-coils: Effects of hydrophobicity and α -helical propensity on protein folding, stability, and specificity. *J Biol Chem* 2002;277:37272–37279. [PubMed: 12138097]
59. Chana M, Tripet BP, Mant CT, Hodges RS. The role of unstructured highly charged regions on the stability and specificity of dimerization of two-stranded α -helical coiled-coils: Analysis of the neck-hinge region of the kinesin-like motor protein Kif3A. *J Struct Biol* 2002;137:206–219. [PubMed: 12064947]
60. Chana MS, Tripet BP, Mant CT, Hodges RS. Stability and specificity of heterodimer formation for the coiled-coil neck regions of the motor proteins Kif3A and Kif3B: The role of unstructured oppositely charged regions. *Journal of Peptide Research* 2005;65:209–220. [PubMed: 15705165]
61. De Crescenzo G, Litowski JR, Hodges RS, O'Connor-McCourt MD. Real-time monitoring of the interactions of two-stranded *de novo* designed coiled-coils: Effect of chain length on the kinetic and thermodynamic constants of binding. *Biochemistry* 2003;42:1254–1263. [PubMed: 12564928]
62. Huyghues-Despointes BM, Baldwin RL. Ion-pair and charged hydrogen-bond interactions between histidine and aspartate in a peptide helix. *Biochemistry* 1997;36:1965–1970. [PubMed: 9047293]
63. Smith JS, Scholtz JM. Energetics of polar side-chain interactions in helical peptides: salt effects on ion pairs and hydrogen bonds. *Biochemistry* 1998;37:33–40. [PubMed: 9425023]
64. Baldwin RL, Rose GD. Is protein folding hierarchic? I Local structure and peptide folding. *Trends Biochem Sci* 1999;24:26–33. [PubMed: 10087919]
65. Lu SM, Hodges RS. A *de novo* designed template for generating conformation specific antibodies that recognize α -helices in proteins. *J Biol Chem* 2002;277:23515–23524. [PubMed: 11971904]
66. Tripet B, Howard MW, Jobling M, Holmes RK, Holmes KV, Hodges RS. Structural characterization of the SARS-Coronavirus spike S fusion protein core. *J Biol Chem* 2004;279:20836–20849. [PubMed: 14996844]
67. Lee DL, Ivaninskii S, Burkhard P, Hodges RS. Unique stabilizing interactions identified in the two-stranded α -helical coiled-coil: Crystal structure of a corexillin I/GCN4 hybrid coiled-coil peptide. *Protein Sci* 2003;12:1395–1405. [PubMed: 12824486]
68. Kammerer RA, Schulthess T, Landwehr R, Lustig A, Engel J, Aepli U, Steinmetz MO. An autonomous folding unit mediates the assembly of two-stranded coiled coils. *Proc Natl Acad Sci USA* 1998;95:13419–13424. [PubMed: 9811815]
69. Steinmetz MO, Stock A, Schulthess T, Landwehr R, Lustig A, Faix J, Gerisch G, Aepli U, Kammerer RA. A distinct 14 residue site triggers coiled-coil formation in cortexillin. *EMBO J* 1998;17:1883–1891.
70. Trybus KM, Freyzon Y, Faust LZ, Sweeney HL. Spare the rod, spoil the regulation: necessity for a myosin rod. *Proc Natl Acad Sci USA* 1997;94:48–52. [PubMed: 8990159]
71. Burkhard P, Kammerer RA, Steinmetz MO, Bourenkov GP, Aepli U. The coiled-coil trigger site of the rod domain of cortexillin 1 unveils a distinct network of interhelical and intrahelical salt bridges. *Structure* 2000;8:223–230. [PubMed: 10745004]
72. Frank S, Lustig A, Schulthess T, Engel J, Kammerer RA. A distinct seven-residue trigger sequence is indispensable for proper coiled-coil formation of the human macrophage scavenger receptor oligomerization domain. *J Biol Chem* 2000;275:11672–11677. [PubMed: 10766786]
73. Lee DL, Lavigne P, Hodges RS. Are trigger sequences essential in the folding of two-stranded α -helical coiled-coils? *J Mol Biol* 2001;306:539–553. [PubMed: 11178912]
74. Kwok SC, Hodges RS. Stabilizing and destabilizing clusters in the hydrophobic core of long two-stranded α -helical coiled-coils. *J Biol Chem* 2004b;279:21576–21588. [PubMed: 15020585]

75. Kwok SC, Hodges RS. Clustering of large hydrophobes in the hydrophobic core of two-stranded α -helical coiled-coils controls protein folding and stability. *J Biol Chem* 2003;278:35248–35254. [PubMed: 12842878]
76. Lu SM, Hodges RS. Defining the minimum size of a hydrophobic cluster in two-stranded α -helical coiled-coils: effects on protein stability. *Protein Sci* 2004;13:714–726. [PubMed: 14978309]
77. Suarez MC, Lehrer SS, Silva JL. Local heterogeneity in the pressure denaturation of the coiled-coil tropomyosin because of subdomain folding units. *Biochemistry* 2001;40:1300–1307. [PubMed: 11170456]
78. Ishii Y, Hitchcock-DeGregori S, Mabuchi K, Lehrer SS. Unfolding domains of recombinant fusion $\alpha\alpha$ -tropomyosin. *Protein Sci* 1992;1:1319–1325. [PubMed: 1303750]
79. Potekhin SA, Privalov PL. Cooperative blocks in tropomyosin. *J Mol Biol* 1982;159:519–535. [PubMed: 7166753]
80. Sturtevant JM, Holtzer ME, Holtzer A. A scanning calorimetric study of the thermally induced unfolding of various forms of tropomyosin. *Biopolymers* 1991;31:489–495. [PubMed: 1868164]
81. Greenfield NJ, Hitchcock-DeGregori SE. The stability of tropomyosin, a two-stranded coiled-coil protein, is primarily a function of the hydrophobicity of residues at the helix-helix interface. *Biochemistry* 1995;34:16797–16805. [PubMed: 8527455]
82. Greenfield NJ, Palm T, Hitchcock-DeGregori SE. Structure and interactions of the carboxyl terminus of striated muscle α -tropomyosin: it is important to be flexible. *Biophysical Journal* 2002;83:2754–2766. [PubMed: 12414708]
83. Greenfield NJ, Swapna GVT, Huang Y, Palm T, Graboski S, Montelione GT, Hitchcock-DeGregori SE. The structure of the carboxyl terminus of striated α -tropomyosin in solution reveals an unusual parallel arrangement of interacting α -helices. *Biochemistry* 2003;42:614–619. [PubMed: 12534273]
84. Brown JH, Kim KH, Jun G, Greenfield NJ, Dominguez R, Volkmann N, Hitchcock-DeGregori SE, Cohen C. Deciphering the design of the tropomyosin molecule. *PNAS* 2001;98:8496–8501. [PubMed: 11438684]
85. Phillips GN, Chacko S. Mechanical properties of tropomyosin and implications for muscle regulation. *Biopolymers* 1996;38:89–95. [PubMed: 8679944]
86. Singh A, Hitchcock-DeGregori SE. Local destabilization of the tropomyosin coiled coil gives the molecular flexibility required for actin binding. *Biochemistry* 2003;42:14114–14121. [PubMed: 14640678]
87. Singh A, Hitchcock-DeGregori SE. Dual requirement for flexibility and specificity for binding of the coiled-coil tropomyosin to its target actin. *Structure* 2006;14:43–50. [PubMed: 16407064]
88. Pato MO, Mak AS, Smillie LB. Fragments of rabbit striated muscle α -tropomyosin. *J Biol Chem* 1981;256:593–601. [PubMed: 7451462]
89. Paulucci AA, Hicks L, Machado A, Miranda MTM, Kay CM, Farah CS. Specific sequences determine the stability and cooperativity of folding of the C-terminal half of tropomyosin. *J Biol Chem* 2002;277:39574–39584. [PubMed: 12167616]
90. Lau SYM, Taneja AK, Hodges RS. Effects of HPLC solvents and hydrophobic supports on the secondary and quaternary structure of a model protein: reversed-phase and size-exclusion high performance liquid chromatography. *J Chromatogr* 1984b;317:129–140.
91. Krylov D, Barchi J, Vinson C. Interhelical interactions in the leucine zipper coiled-coil dimer: pH and salt dependence of coupling energy between charged amino acids. *J Mol Biol* 1998;279:959–972. [PubMed: 9642074]
92. Burkhard P, Ivaninskii S, Lustig A. Improving coiled-coil stability by optimizing ionic interactions. *J Mol Biol* 2002;318:901–910. [PubMed: 12054832]
93. Huyghues-Despointes BM, Scholtz JM, Baldwin RL. Helical peptides with three pairs of Asp-Arg and Glu-Arg residues in different orientations and spacings. *Protein Sci* 1993;2:80–85. [PubMed: 8443591]
94. Spek EJ, Bui AH, Lu M, Kallenbach NR. Surface salt bridges stabilize the GCN4 leucine zipper. *Protein Science* 1998;7:2431–2437. [PubMed: 9828010]
95. Hitchcock-DeGregori SE, Song Y, Greenfield NJ. Functions of tropomyosin's periodic repeats. *Biochemistry* 2002;41:15036–15044. [PubMed: 12475253]

96. Lehrer SS, Yuan A. The stability of tropomyosin at acid pH: effects of anion binding. *J Struct Biol* 1998;122:176–179. [PubMed: 9724618]
97. Gooding C, Reinach FC, Macleod AR. Complete nucleotide sequence of the fast-twitch isoform of chicken skeletal muscle α -tropomyosin. *Nuc Acid Res* 1987;15:8105–8105.
98. Mills JB, Mant CT, Hodges RS. One-step purification of recombinant proteins from a whole cell extract by reversed-phase high-performance liquid chromatography. *J Chromatogr A* 2006;113:248–253. [PubMed: 16945380]
99. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990;215:403–410. [PubMed: 2231712]
100. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997;25:3389–3402. [PubMed: 9254694]
101. Monteiro PB, Lataro RC, Ferro JA, DeCastro Reinach F. Functional alpha-tropomyosin produced in *Escherichia coli*. A dipeptide extension can substitute the amino-terminal acetyl group. *J Biol Chem* 1994;269:10461–10466. [PubMed: 8144630]
102. Meier M, Burkhard P. Statistical analysis of intrahelical ionic interactions in α -helices and coiled-coils. *J Structural Biol* 2006;155:116–129.

	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>	<i>g</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>	<i>g</i>	
1	Met	Asp	Ala	Ile	Lys	Lys	Lys	Met	Gln	Met	Leu	Lys	Leu	Asp	14
15	Lys	Glu	Asn	Ala	Leu	Asp	Arg	Ala	Glu	Gln	Ala	Glu	Ala	Asp	28
29	Lys	Lys	Ala	Ala	Gln	Glu	Arg	Ser	Lys	Gln	Leu	Glu	Asp	Gln	42
43	Leu	Val	Ala	Leu	Gln	Lys	Lys	Leu	Lys	Gln	Thr	Glu	Asp	Gln	56
57	Leu	Asp	Lys	Tyr	Ser	Glu	Ser	Leu	Lys	Asp	Ala	Gln	Gln	Lys	70
71	Leu	Glu	Leu	Ala	Asp	Lys	Lys	Ala	Thr	Asp	Ala ⁸¹	Glu	Ser	Gln	84
85	Val	Ala	Ser	Leu	Asn	Arg	Arg	Asn ⁹²	Gln	Leu	Val	Gln	Gln	Gln	98
99	Leu ⁹⁹	Asn	Arg	Ala	Gln	Glu	Arg	Leu	Ala	Thr	Ala	Leu	Gln	Lys	112
113	Leu	Glu	Gln	Ala	Gln	Lys	Ala ¹¹⁷	Ala	Asp	Gln	Ser	Glu	Arg	Gly	126
127	Met	Lys	Val	Ile	Gln ¹³¹	Asn	Arg	Ala	Gln	Lys	Asp	Glu	Gln	Lys	140
141	Met	Glu	Ile	Gln	Gln	Ile	Gln	Leu	Lys	Gln	Ala	Lys	His	Ile	154
155	Ala	Glu	Gln	Ala	Asp	Arg	Lys	Tyr	Gln	Gln	Ala	Ala	Arg	Lys	168
169	Leu	Val	Ile	Ile	Gln	Gly	Asp	Leu	Gln	Arg	Ala	Glu	Gln	Arg	182
183	Ala	Glu	Leu	Ser	Gln	Ser	Lys	Cys	Ala	Gln	Leu	Gln	Gln	Gln	196
197	Leu	Lys	Thr	Val	Thr	Asn	Asn	Leu	Lys	Ser	Leu	Glu	Ala	Gln	210
211	Ala	Glu	Lys	Tyr	Ser	Gln	Lys	Glu	Asp	Lys	Tyr	Gln	Gln	Gln	224
225	Ile	Lys	Val	Leu	Thr	Asp	Lys	Leu	Lys	Gln	Ala	Gln	Thr	Arg	238
239	Ala	Glu	Phe	Ala	Gln	Arg	Ser	Val	Thr	Lys	Leu	Gln	Lys	Ser	252
253	Ile	Asp	Asp	Leu	Gln	Asp	Gln	Leu ²⁶⁰	Tyr	Ala	Gln	Lys	Leu	Lys	266
267	Tyr	Lys	Ala	Ile	Ser	Glu	Gln	Leu	Asp	His	Ala	Leu	Asn	Asp	280
281	Met	Thr	Ser	Ile ²⁸⁴											

Fig. 1. Amino acid sequences of the chicken skeletal α -tropomyosin

Heptad positions (*a* to *g*) are denoted above the sequence, and the core positions *a* and *d* are shaded grey. Stabilizing clusters (three or more consecutive large hydrophobic residues in the *a* and *d* positions, consisting of Leu, Ile, Val, Met, Phe, Tyr) are designated by a solid-line rectangular box. Destabilizing clusters (three or more consecutive destabilizing residues in the *a* and *d* positions, consisting of Gly, Ala, Cys, Asp, Glu, Asn, Gln, Ser, Thr, His, Lys, Arg) are denoted by a dashed-line rectangular box. The regions not designated by boxes are intervening regions which contain a mixture of stabilizing and destabilizing *a* and *d* residues that are not in clusters. Underscored residues with superscripted sequence numbers denote the C-terminal residue of the TM fragments studied.

Fig. 2. A stability profile of chicken skeletal tropomyosin

A 14-residue window was used to calculate the sum of α -helical propensity for residues in positions **b**, **c**, **e**, **f** and **g** and the stability coefficients for positions **a** and **d**^{14, 15}. The algorithm STABLECOIL

(<http://biomol.uchsc.edu/researchFacilities/ComputationalCore/stablecoil/index.html>).

Fig. 3. Representative CD spectra of the two TM fragments (panel A: 1-99 and panel B: 1-119) at 20°C

CD experiments were carried out in a 50 mM PO_4 buffer with 100 mM KCl, pH 7.0, 2 mM DTT (benign buffer). Solid diamonds denote benign buffer and opened diamonds denote benign buffer containing 50% trifluoroethanol (v/v). Concentrations of TM fragments were approximately 5 μM (monomer).

Fig. 4. Thermal denaturation profiles of full length TM and TM fragments

Panel A shows the denaturation curves of TM fragments and full-length TM in benign buffer (50 mM PO_4 , 100 mM KCl, pH 7.0 containing 2 mM DTT). Concentrations ranged from 3–5 μM monomer. Panel B plots the correlation between temperature midpoint (stability) versus the size of TM fragment. The open circles show the dramatic increase in protein stability by the addition of residues 100–119. The solid diamonds denote the temperature midpoint values.

Figure 1: Schematic representation of the protein structure and electrostatic clusters. The protein sequence is shown at the top with residues 85 to 119. Below, two rows of residues are shown: (V)A S (L)N R R (I)Q L (V)E and (V)A S (L)N R R (I)Q L (V)E. Electrostatic Cluster 1 (97-104) is highlighted with a dashed box, containing residues E E (L) D R (A) Q E. Electrostatic Cluster 2 (112-118) is highlighted with a dashed box, containing residues K (L) E E A E K A. Arrows indicate interactions between residues in the two clusters.

Fig. 5. Amino acid sequence of TM 85–119 showing stabilizing ionic interactions
Residues at heptad positions *a* and *d* (hydrophobic core) are denoted by circles (stabilizing residues) or by boxes (destabilizing residues). Intrachain ionic attractions are denoted by brackets and interchain ionic attractions are denoted by intersecting arrows. The proposed stability control sites 97–104 and 112–118 are denoted by dashed rectangular boxes. The arrow above and below residue 110 denote the Leu in position *e* that has been previously shown to affect stability⁶⁷.

Fig. 6. The heptad occurrence in a tropomyosin data base

The data base contains 379 sequences and 13,647 heptads of which 2,144 are unique heptad sequences.

Fig. 7. Thermal denaturation profiles of TM fragments

Panel A shows the denaturation curves of TM fragments 1–99 ($T_m=29.2^\circ\text{C}$), 1–105 ($T_m=33.1^\circ\text{C}$), 1–110 ($T_m=37.1^\circ\text{C}$) and 1–119 ($T_m=47.2^\circ\text{C}$) in benign buffer (50 mM PO_4 , 100 mM KCl, pH 7.0) ranging from 32–52 μM monomer. Panel B shows the temperature denaturation profiles of TM 1–119 at 5 concentrations of monomer; 52 μM ($T_m=47.2^\circ\text{C}$), 16 μM ($T_m=45.7^\circ\text{C}$), 3.1 μM ($T_m=43.3^\circ\text{C}$), 0.63 μM ($T_m=42.3^\circ\text{C}$), and 0.31 μM ($T_m=41.6^\circ\text{C}$).

Table 1
Biophysical Data for the two-stranded TM fragments

TM Fragment	1-81	1-92	1-99	1-119	1-131	1-260	1-284
$[\theta]_{222}$ (benign) ^a	-25,900	-26,100	-25,600	-29,900	-30,200	-30,500	-30,300
$[\theta]_{222}$ (50% TFE) ^a	-33,000	-32,900	-33,100	-34,200	-33,800	-33,600	-33,700
$[\theta]_{222}/[\theta]_{208}$ ^b	1.02	1.02	1.04	1.12	1.08	1.06	1.07
T _m (°C) ^c	28.5	26.5	26.0	42.0	43.0	40.0	43.0
Molecular Mass ^d	9223	10,479	11,320	13,457	14,801	29,886	32,651
Molecular Mass ^e	12,397 (M-D)	15,526 (M-D)	19,843 (M-D)	24,655 (D)	29,923 (D)	59,494 (D)	67,686 (D)

^a $[\theta]_{222}$ is the mean residue molar ellipticity ($\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$) measured at 222 nm in 100 mM KCl, 50 mM PO₄, (K₂HPO₄/KH₂PO₄) buffer, pH 7.0, containing 2 mM DTT in the absence (benign) and presence of 50% trifluoroethanol (50% TFE, v/v). Concentrations of TM fragments ranged from 3–5 μM monomer.

^bThe ratio $[\theta]_{222}/[\theta]_{208}$ was calculated by dividing the observed molar ellipticity value at 222 nm by the observed molar ellipticity value at 208 nm in benign buffer.

^cT_m is the temperature at which there is a 50% decrease in fraction folded compared to the fully folded coiled-coil as determined by circular dichroism.

^dThe calculated mass of each fragment monomer is shown.

^eThe apparent mass observed by sedimentation equilibrium experiments by analytical ultracentrifugation. (M-D) denotes a monomer-dimer equilibrium. (D) denotes predominantly dimer molecular mass from single species fit.

Table 2

Heptad Sequences and their Occurrence in Tropomyosin

Residue #	Sequence ^a						Number of Charged Residues	Number and Type of Electrostatic Attractions				Total Occurrence in TM Sequences ^b
	g	a	b	c	d	e	f	Intrachain i to i+3	Intrachain i to i+4	Interchain i to i+5	Total Number of Attractions	
91–97	R	I	Q	L	V	E	E	0	0	1	1	239
98–104	E	L	D	R	A	Q	E	2	0	0	2	244
105–111	R	L	A	T	A	L	Q	0	0	0	0	221
112–118	K	L	E	E	A	E	K ^c	2	1	1	4	248
119–125	A	A	D	E	S	E	R	1	1	0	2	294
147–153	Q	L	K	E	A	K	H	1	0	0	1	231
161–167	K	Y	E	E	V	A	R	2	1	0	3	245
217–223	K	E	D	K	Y	E	E	1	0	1	2	208
231–237	K	L	K	E	A	E	T	2	0	1	3	223
238–244	R	A	E	F	A	E	R	0	1	1	2	277
112–118	K	L	E	E	A	E	K ^c	2	1	1	4	248
1	K	L	K	E	A	E	E	2	1	1	4	0
2	E	L	E	E	A	K	K	2	1	1	4	0
3	E	L	E	K	A	K	E	3	0	1	4	1
4	E	L	K	K	A	E	E	3	1	0	4	0
5	K	L	E	K	A	E	E	1	0	1	2	0
6	E	L	K	E	A	K	E	0	1	1	2	0
7	E	L	E	K	A	E	K	1	1	0	2	0
8	E	L	K	E	A	E	K	2	0	0	2	0
9	K	L	E	E	A	K	E	2	0	0	2	0

^aThe top panel has the 10 highest occurring heptad sequences found in tropomyosins (see Fig. 6). The bottom panel shows 9 sequences with the same hydrophobic core as the “stability control site” (112–118) at positions **a** and **d** and the same composition but different sequence.

^bThere are 379 different tropomyosin sequences in our data base and a total of 13,647 heptads and 2,144 unique heptad sequences (see Fig. 6 and methods section).

^c“stability control site” identified in this study (position 112–118 in chicken skeletal tropomyosin).

Table 3

Heptad sequences in tropomyosins that have interchain i to $i' + 5$, intrachain i to $i + 3$ and i to $i + 4$ salt bridges with homology to KLEEA EK

Sequence #	g	a	b	c	d	e	f	Total Occurrence in TM Sequences	Region of TM
1	K	L	E	E	A	E	K	248	1
2	K	L	D	E	A	E	K	1	1
3	K	L	E	E	T	E	K	3	1
4	K	L	E	E	L	E	K	2	1
Subtotal								254	
5	K	L	D	D	A	E	K	1	2
6	K	L	E	E	K	E	K	15	2
7	K	L	D	E	K	E	K	1	2
8	K	L	D	E	K	D	K	4	2
9	K	L	E	E	K	D	K	9	2
10	K	L	E	E	T	E	K	5	2
11	K	L	E	E	T	D	K	4	2
12	K	M	E	E	T	D	K	1	2
Subtotal								40	
Total								294	

^aRegion 1 is defined by homology to the 35-residue sequence RIQLVEEELDRAQERLATALQ**KLEEA**EKAADSEER which is analogous to 91–125 in the 284 residue tropomyosin sequences.

Region 2 is defined by homology to the 42-residue sequence KMQQVENELDQVQEQLSLANT**KLEEKEK**ALQNAEGEVAALNR which is analogous to 49–90 in the 284 residue tropomyosin sequences.