

## Stability Control Sites in Two-stranded $\alpha$ -Helical Coiled-Coils

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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. This objective is paramount since it has been observed that even a single mutation in the sequence of a protein can dramatically alter function and lead to a disease state. Obviously, a threshold stability is required to initiate protein folding. Therefore, characterizing all of the complex inter-residue interactions involved in determining protein stability is key to understanding conformational change in proteins and their function.

To reduce complexity in our study of protein stability, we chose to study one of nature's most simplistic protein motifs, the two-stranded  $\alpha$ -helical coiled-coil. The major advantage of this motif is its rod-like nature which simplifies protein folding and stability to a one dimensional problem rather than the three dimensional problem observed in globular proteins. The protein under investigation here is tropomyosin, a parallel, two-stranded  $\alpha$ -helical coiled-coil containing 284 residues per polypeptide chain.

Our laboratory has previously shown that the stability of long coiled-coils is controlled by alternating clusters of stabilizing and destabilizing residues in the hydrophobic core [1,2]. We have also shown that the effect of chain length on coiled-coil stability depends on the stabilizing or destabilizing nature of the heptads added to or removed from a coiled-coil [3]. The questions that we wanted to address in this study, are 1) 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 or 2) whether there is a unique site(s) that does not control protein folding but does control the final stability of the protein.

### Results and Discussion

To verify the existence of such a site, we prepared a series of C-terminal deletion fragments of tropomyosin (1-81, 1-92, 1-99, 1-119, 1-131, and 1-260) and examined their folding and stability. All constructs were fully folded, two-stranded  $\alpha$ -helical coiled-coils. However, thermal denaturation profiles for tropomyosin and the C-terminal deletion fragments revealed a stability difference ( $\Delta T_m$ ) of 15°C between fragments 1-99 and 1-119 (not shown). Figure 1 shows a plot of this data and indicates that native, wild type stability ( $T_m = 43^\circ\text{C}$ ) was not achieved until a length of 119 residues was reached. Fragments 1-81, 1-92, and 1-99 had  $T_m$  values of 26-28.5°C, whereas all other fragments (1-119, 1-131, and 1-260) had  $T_m$  values of 40-43°C. Inspection of the sequence between residues 97-119 shows that the

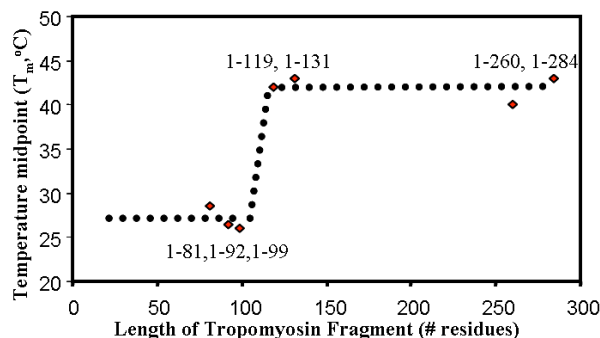


Figure 1. Plotted  $T_m$  values for tropomyosin and C-terminal deletion fragments

hydrophobic core contains three destabilizing Ala residues (positions 102, 109, and 116) (Figure 2) all of which are predicted to destabilize the coiled-coil upon extending the sequence from 1-99 to 1-119 [3-6]. Thus, whatever is responsible for the enhanced stability in the region 97-119, must overcome this destabilization in the core and add stability as well. What is immediately obvious is the presence of two unique clusters of electrostatic attractions that may control native protein stability (Figure 2). Also of interest is the presence of a Leu residue at position 110e that could add stability [7]. Interestingly, these two electrostatic clusters and the heptad containing Leu 110 are highly conserved among tropomyosin sequences. We compiled a data base of 379 different tropomyosin sequences composed of a total of 13,647 heptads. A search through our data base found 2144 unique heptad sequences among which electrostatic cluster 1 occurs 244 times, electrostatic cluster 2 occurs 248 times, and the heptad sequence containing Leu 110 occurs 221 times (Figure 3).

To investigate the significance of these sites in controlling protein stability, we prepared a series of synthetic peptide coiled-coil analogs as shown in Figure 2. The synthetic peptides contained the native tropomyosin sequence for residues 85-119 with the addition of an N-terminal CGG linker to form disulfide bridged parallel two-stranded  $\alpha$ -helical coiled-coils independent of the monomer-dimer equilibrium. In addition to the native coiled-coil, five analogs were prepared containing R101A, L110A, R101A/L110A, K112A/K118A, and the quadruple substitution analog R101A/L110A/K112A/K118A. Each substitution eliminates one or more of the electrostatic interactions that contribute to coiled-coil stability, including intra-chain  $i$  to  $i+3$  and  $i$  to  $i+4$  electrostatic interactions and inter-chain  $i$  to  $i'+5$  electrostatic interactions. For example, the R101A analog removes all of the possible intra-chain electrostatic interactions in Cluster 1, while the K112A/K118A analog removes all of

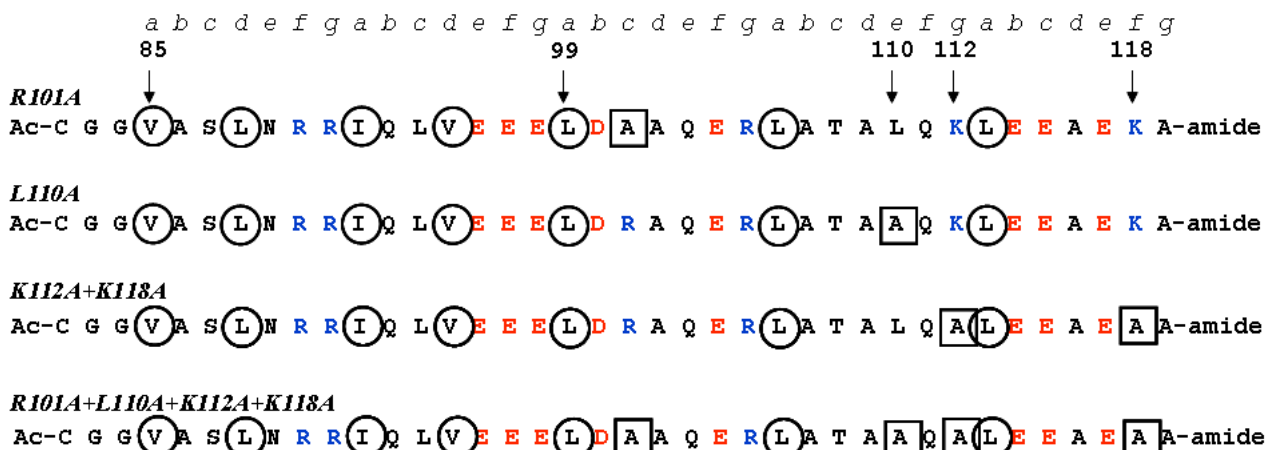
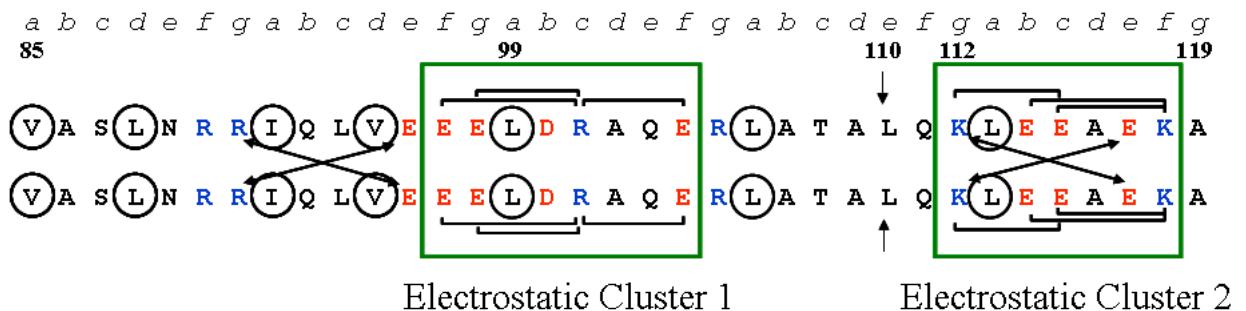


Figure 2. Tropomyosin sequence of interest and synthesized analogs. Electrostatic Clusters 1 and 2 are boxed and labeled. Brackets indicate potential intra-chain electrostatic interactions. Arrows denote inter-chain electrostatic interactions. Circled residues denote large hydrophobes in the hydrophobic core a and d positions. Boxed residues highlight substitution sites.

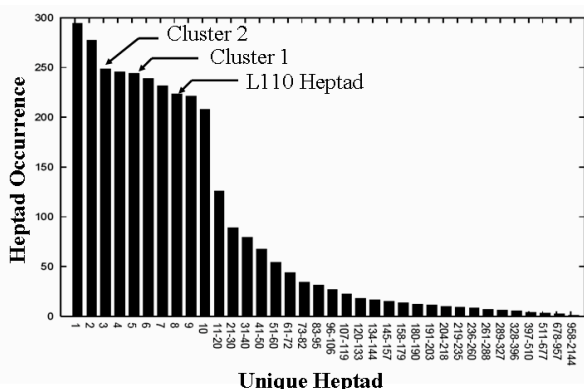


Figure 3. Frequency of Conserved Heptad Occurrence.

the intra-chain and inter-chain electrostatic interactions in Cluster 2.

In order to assess changes in coiled-coil stability resulting from the loss of these interactions, we analyzed the synthesized peptides by circular dichroism (CD). CD spectra measured at 5°C (not shown) revealed that the disulfide bridged coiled-coil L110A is a fully folded two-stranded coiled-coil in benign conditions (100mM KCl, 50mM PO<sub>4</sub>, pH 7) with no further α-helical structure induced by the addition of 50% TFE. Similarly, the analogs R101A, K112A/K118A, and the native coiled-coils were also found to be fully folded. However, the R101A/L110A analog and the quadruple substituted analog are only partially folded in benign conditions compared to their observed α-helical content in 50% TFE.

Table 1. Thermal stability data for native tropomyosin sequence 85-119 and analogs.

Sequence	T <sub>m</sub> (C°)	ΔT <sub>m</sub> (C°)
Native	52.9	---
R101A	46.0	-6.9
K112A+K118A	45.3	-7.6
L110A	27.9	-25.0
R101A+L110A	< 5	---
R101A+L110A+K112A+K118A	< 5	---

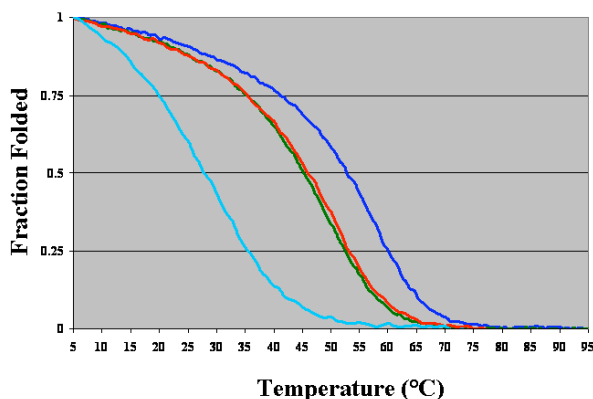


Figure 4. Thermal denaturation profiles of native tropomyosin sequence 85-119 (blue) and analogs R101A (red), K112A+K118A (green), and L110A (light blue).

Table 1 summarizes the data obtained from the thermal denaturation profiles of the native synthetic coiled-coil and three analogs shown in Figure 4. Removing all of the electrostatic interactions from Cluster 1 resulted in a 6.9°C reduction in thermal stability. Removal of all of the electrostatic interactions from Cluster 2 resulted in a 7.6°C reduction in thermal stability when compared to the native sequence. Interestingly, removal of the hydrophobic residue L110 (L110A), which is in an e position of the coiled-coil, resulted in a 25.0°C decrease in stability, the largest decrease compared to the native sequence. The  $T_m$  values for the double substituted analog, R101A/L110A, and the quadruple substituted analog are less than 5°C since these coiled-coils are less than 50% folded at 5°C.

Based on previously reported chain length [3] and stability studies [4,5,6], extending the polypeptide chain length in tropomyosin from 1-99 to 1-119 was expected to be tremendously destabilizing since three alanine residues were present in the hydrophobic core positions 102, 109, and 116. Instead, we observed the dramatic increase in stability reflected in Figure 1. This suggests the presence of other interactions that not only overcome the destabilizing effects of the alanine residues in the hydrophobic core but also dramatically increase final stability of the protein. We examined electrostatic clusters 1 and 2 because of their unique composition of five charged residues out of the eight present in cluster 1 and five charged residues out of seven in cluster 2. It had been previously shown that Leu residues in positions e and g of coiled-coils can contribute to protein stability [7]. What is interesting is that Leu 110 makes a greater than 3-fold contribution to stability than either of the electrostatic clusters. The relative contribution to stability is Leu 110 ( $\Delta T_m = 25.0^\circ\text{C}$ ) > Cluster 2 ( $\Delta T_m = 7.6^\circ\text{C}$ )  $\approx$  Cluster 1 ( $\Delta T_m = 6.9^\circ\text{C}$ ). Obviously, the combined effects of these three stability sites, all located in the 22 residue region 97-119, can dramatically increase the overall stability of the 1-119 coiled-coil even with the destabilizing effect of the three Ala residues in the hydrophobic core. The contribution of Leu 110 to coiled-coil stability is dramatically larger than previously reported [7]. This suggests that it must pack uniquely in the hydrophobic core despite being in an e position. Structural analysis of this coiled-coil sequence should reveal a unique packing arrangement not previously observed. In conclusion, we have identified for the first time a unique stability control region in the two-stranded  $\alpha$ -helical coiled-coil, tropomyosin. The question remains, what is the functional role of this unique region in tropomyosin during muscle regulation?

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