Conserved Asp-137 Imparts Flexibility to Tropomyosin and Affects Function*

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Tropomyosin (Tm) is an α-helical coiled-coil that controls muscle contraction by sterically regulating the myosin-actin interaction. Tm moves between three states on F-actin as either a uniform or a non-uniform semi-flexible rod. Tm is stabilized by hydrophobic residues in the “a” and “d” positions of the heptad repeat. The highly conserved Asp-137 is unusual in that it introduces a negative charge on each chain in a position typically occupied by hydrophobic residues. The occurrence of two charged residues in the hydrophobic region is expected to destabilize the region and impart flexibility. To determine whether this region is unstable, we have substituted hydrophobic Leu for Asp-137 and studied changes in Tm susceptibility to limited proteolysis by trypsin and changes in regulation. We found that native and Tm controls that contain Asp-137 were readily cleaved at Arg-133 with $t_{1/2}$ of 5 min. In contrast, the Leu-137 mutant was not cleaved under the same conditions. Actin stabilized Tm, causing a 10-fold reduction in the rate of cleavage at Arg-133. The actin-myosin subfragment S1 ATPase activity was greater for the Leu mutant compared with controls in the absence of troponin and in the presence of troponin and Ca$^{2+}$. We conclude that the highly conserved Asp-137 destabilizes the middle of Tm, resulting in a more flexible region that is important for the cooperative activation of the thin filament by myosin. We thus have shown a link between the dynamic properties of Tm and its function.

Tropomyosin (Tm)3 is a coiled-coil α-helix whose isoforms function in several ways when bound to F-actin. In skeletal and cardiac muscle, Tm controls contraction by regulating the binding of myosin to actin in conjunction with tropinin (Tn) and Ca$^{2+}$ (1). Tm is also involved in the myosin-induced cooperative activation of the thin filament (2). In smooth muscle, Tm is involved in the cooperative activation of contraction by phosphorylated myosin (3). Non-muscle Tm stabilizes actin filaments and modulates the binding of other proteins (4). Equilibria between three thin filament states, B (blocked), C (closed or Ca$^{2+}$-induced), O or M (open or myosin-induced), are involved in the regulation of muscle contraction (5). Ca$^{2+}$ binding to Tn facilitates the initial binding of myosin heads in the first step. In the second step, myosin heads cooperatively facilitate the movement of Tm into the O-state to allow isomerization of the heads and the generation of force. There are several single site Tm mutations that are involved in familial hypertrophic cardiomyopathy (6–10) that increase the Ca$^{2+}$ sensitivity, indicating a shift into the B-state. However, the link between these mutations and the functional changes associated with familial hypertrophic cardiomyopathy is unknown.

Coiled-coils are primarily stabilized by inter-helical hydrophobic interactions in positions a and d in the 7-residue pseudo repeat, a-to-g. Residues at a- and d-positions consist mainly of Leu, Ala, Leu, Val, Tyr, and Met. At position 137 in both α- and β-Tm there is an Asp in a d-position instead of the typical hydrophobic residue. The remaining 24 Asp residues in Tm are outside of the hydrophobic ridge. Asp-137 is conserved in all skeletal and smooth muscle Tms and Tm from Drosophila to humans. Also, for non-muscle Tms, there is an Asp in an equivalent d-position. Asp residues with a short negatively charged side chain located at equivalent d-positions on both chains would be expected to destabilize the coiled-coil due to electrostatic repulsion. To determine the role of Asp-137 in a hydrophobic position, we substituted a Leu for the Asp in recombinant chicken skeletal α-Tm. In a preliminary study we determined that the Leu substitution increased the local and global thermal stability (11). Here we report that tryptic cleavage occurs rapidly at Arg-133 for the native protein and that Leu substitution for Asp-137 markedly inhibits this process, indicating a local instability in native Tm in this region. F-actin inhibits the rate of Tm cleavage 10-fold, but proteolysis occurs in the same place, Arg-133. Although Asp-137 locally destabilizes the coiled-coil, it does not affect the binding to actin. However, the actin-myosin subfragment-1 (S1) ATPase activity is greater for the Leu mutant than for its control, C190A, in the absence of troponin and in the presence of troponin and Ca$^{2+}$. This study provides evidence for the importance of structural flexibility due to a local unstable region that controls the function of Tm in its regulation of muscle activity.

EXPERIMENTAL PROCEDURES

Protein Preparation—All the Tms used in this work are recombinant Tms that have an Ala-Ser N-terminal extension, mimicking the N-terminal acetylation of native Tm (12). To facilitate manipulation of the gene, a C190A Tm DNA construct corresponding to the TPM1 gene for chicken skeletal tropomyosin (Reinach laboratory, Ref. 12) was inserted...
between a BamH1 and Nde1 restriction site in a PAED4 plasmid vector. This vector was then used as a template for the D137L/C190A clone. Because both D137L/C190A and C190A lack the native cysteine at 190, complications due to potential disulfide cross-linking were avoided. The effects of the Leu substitution in D137L/C190A were controlled for by comparing its function with C190A and WT (Cys-190), which retains the native Cys at position 190. Point mutations for these controls and the D137L/C190A Tm were performed by modifying the C190A template using the QuikChange procedure (Stratagene) and confirmed by sequence analysis.

BL21 Escherichia coli cells were transformed using the PAED4 plasmid. Bacterial colonies were grown on ampicillin plates, and individual colonies were selected and used to inoculate a starter culture, which was grown overnight. Plasmid DNA was isolated and purified (Qiagen Miniprep) from samples of the overnight culture. BamH1 and Nd11 restriction nucleases were used to excise the gene insert and visualized on a 1.2 mol ratio of S1 and F-actin in the presence of 5 mM ATP and recovering the supernatant.

Physical characterization of S1, actin, Tm, and Tm was performed in a number of ways. N-terminal sequence analysis was performed to determine intact N-terminal sequence of all recombinant Tms and confirmed by MALDI-TOF mass spectrometry and SDS-PAGE. CD unfolding studies indicated that all recombinant Tms were folded properly. Tm to F-actin binding was confirmed by co-sedimentation and by titrations of actin-S1 ATPase with Tm (19).

ATPase Measurements—ATPase measurements were done on a Cary 50 UV-visible spectrometer using the Enzcheck kit (Invitrogen/Molecular Probes) that couples the release of P_i from ATP to an enzyme-substrate system producing a continuous change in absorbance at 360 nm (20). For Tm binding studies, inhibition of actin-S1 ATPase was monitored during additions of Tm (19, 21). The Ca^{2+} dependence of actin-S1 ATPase for the mutants in the presence of troponin was performed using a 1 mM EGTA/NTA buffer system containing 20 mM MOPS, 5 mM MgCl_2, 25 mM NaCl, pH 7.2; the effective ionic strength was calculated to be 80 or 100 mM for measurements performed in the absence and presence of Ca^{2+} + EGTA/NTA, respectively. Stock calibrated Ca^{2+} solutions were prepared and equilibrium constants (22) corrected to the experimental temperature (23) and ionic strength, and Maxchelator was used to calculate the free Ca^{2+} concentration. The pCa_{50} was determined by fitting the ATPase dependence on the Ca^{2+} concentration to the Hill equation using Kaleidegraph (Synergy Software). Errors were estimated from the standard deviation of replicate runs.

Trypsin Digestion and Identification of Products—Tm in HEPES buffer (0.5 mg/ml in 0.1 M NaCl, 5 mM MgCl_2, 10 mM β-mercaptoethanol, 10 mM HEPES buffer, pH 7.2) was treated with 0.002 mg/ml porcine trypsin (MSG; GE Biosciences) in a 30 °C water bath. At various times, aliquots were removed and mixed with 40× excess soybean trypsin inhibitor to quench the reaction. A control experiment was done to ensure that soybean trypsin inhibitor efficiently quenched the reaction. The samples were then prepared for SDS gel electrophoresis and mass spectral analyses. For Tm bound to actin, Tm at 0.3 mg/ml was reacted in the absence and presence of 1.5 mg/ml F-actin for increasing times in buffer. The trypsin-treated samples were diluted 1:10 with sinnapinic acid matrix and subjected to mass spectral analyses using a Perceptive Voyager MALDI-TOF.

For N-terminal amino acid analyses, SDS-PAGE protein bands of trypsin-treated samples were blotted onto mini Pro-Blott polyvinylidene difluoride-type membranes (Applied Biosciences), extracted, and sequenced with standard protocols using an Applied Biosciences Procise sequencing instrument. The Procise software package was used to identify the first 10 N-terminal residues. Densitometry was performed on SDS gels using the fluorescence of the Coomassie stain, imaged with the
Odyssey system (excited at 700 nm), and analyzed with the Odyssey software (version 2.0.4.0).

**Structural Analysis**—The hydrophobic packing was compared at Asp-137 and Leu-207 in the 2.3 Å rat Tm structure (Protein Data Bank code 2b9c) (24) using PyMOL (DeLano Scientific, Palo Alto, CA).

**RESULTS**

**Proteolytic Digestion of Tm**—The Leu mutant D137L/C190A, a non-Cys control (C190A), and a wild type (WT) Tm were used in the digestion studies. Limited tryptic digestion of the Asp-137-containing controls C190A and WT initially produced two fragments, each about half the molecular weight of the intact Tm (Fig. 1). Previous studies identified two fragments, an N-terminal fragment, T1, and a C-terminal fragment, T2, due to initial tryptic cleavage of rabbit skeletal αα-Tm at Arg-133 (25). Preliminary studies with native Rana temporaria muscle Tm showed that αα-Tm also was cleaved at Arg-133 (data not shown). The same site of cleavage was identified for these control recombinant chicken αα-Tms (see below). We also noted that some truncation (cleavage at the ends) of the Tms occurred as observed by a slightly faster moving band in SDS gels. SDS gels were used to monitor the loss of the main Tm band (intact and truncated) and to determine the kinetics of initial cleavage at Arg-133. For both the WT and C190A controls, the loss of the main band occurred with a $t_{1/2}$ of ~5 min (Fig. 1C). T1 and T2 were initially produced and were cleaved into smaller peptides with time. In contrast, the Leu mutant D137L/C190A was not cleaved at Arg-133 during a 1-h incubation. However, the Leu mutant was successively truncated by trypsin as indicated by the appearance of slightly faster migrating species (Fig. 1A). The molecular mass and the sites of cleavage and truncation were determined by mass spectrometry (Fig. 2) and N-terminal analyses (Fig. 3); see below.

**Sites of Tm Proteolysis by Trypsin**—MALDI-TOF mass spectra of intact Tm showed a main band of the single chain at 33 kDa and a band of the two-chain molecule at 66 kDa (not shown) of much lower intensity. Therefore, the laser ionization procedure did not completely dissociate the molecule. Mass spectra were obtained for each time point of digestion. Here we show only molecular masses below 35 kDa of C190A at 3 and 6 min. At 3 min, in addition to the main band and the soybean trypsin inhibitor band (STI) (at 20 kDa), peptides T1 and T2 are seen (Fig. 2A). At 6 min, some remaining intact Tm chains are present (peak 1 as well as some truncated Tm chains, peak 2. Peak (T2)2 appears to be the presence of some dimer chains of peptide T2, indicating that T2 can exist as a dimer in solution, in agreement with earlier CD studies (25). The dimer of T1 may also be present, but because of sample heterogeneity (truncated and intact species) and overlap with Peak 1, it was not positively identified. The indicated molecular mass helps define the peptides produced when compared with N-terminal analyses (see below). The MS spectra of D137L/C190A treated with trypsin for short and long times show a different pattern (Fig. 2B). At 6 min, some remaining intact Tm chains are present (peak 1 as well as some truncated Tm chains, peak 2. Peak (T2)2 appears to be the presence of some dimer chains of peptide T2, indicating that T2 can exist as a dimer in solution, in agreement with earlier CD studies (25). The dimer of T1 may also be present, but because of sample heterogeneity (truncated and intact species) and overlap with Peak 1, it was not positively identified. The indicated molecular mass helps define the peptides produced when compared with N-terminal analyses (see below). The MS spectra of D137L/C190A treated with trypsin for short and long times show a different pattern (Fig. 2B). At 6 min, almost equal quantities of intact Tm chains and truncated chains are present. The difference in molecular mass is ~1000 Da, which corresponds to 9–10 amino acid residues clipped off (see below for identification of site). After 60 min of treatment,
two other truncated species of lower molecular mass appeared. No significant cleavage to produce T1 and T2 is observed in the presence of actin reduced the rate of cleavage of Tm by a factor of 10 (Fig. 1D). F-actin alone was not affected by the 1 h of incubation with trypsin. Mass spectra showed that although Tm on actin-Tm produced some T1 and T2 peptides, there was no truncation at Lys-7. This suggested that F-actin protected Tm from truncation, in agreement with the known 9–11 amino acid overlap of the N and C termini (26) and the recent structure of the overlap region (27), which would protect cleavage at Lys-7. The lower rate of cleavage while bound to F-actin (Fig. 1D) indicates that actin stabilizes Tm. However, the production of T1 and T2 indicates that cleavage at Arg-133 still takes place while Tm is bound to actin. The Leu mutant was not cleaved or truncated while bound to actin during the same time period as the controls, as expected.

Functional Effects of D137L Mutation—Actin binding properties of the Tms were studied by co-sedimentation with F-actin and also by titrations of F-actin S1 ATPase activity with Tm (19) using the continuous phosphate colorimetric assay system (see “Experimental Procedures”). F-actin co-sedimentation studies showed that for the Leu mutant and controls no Tm was found in the supernatant when 1 µM Tm was mixed with 10 µM actin (10 mM HEPES buffer, pH 7.5, 5 mM MgCl2, 30 mM NaCl) and spun to pellet the actin. Tm titrations of the F-actin-S1 ATPase at low [S1] showed that the Leu mutant D137L/C190A binds to actin with similar binding constant, Kd = 2 × 107 M–1 (data not shown).

To determine the effect of the more stable Leu mutant on the Ca2+-dependent ATPase of the fully regulated thin filament actin Tm-Tn-S1, we used the continuous phosphate assay system (20). At high Ca2+, the ATPase was activated almost 2-fold more for the Leu mutant than the control (Fig. 4A). The S1 dependence of the actin-Tm-S1 ATPase (4B) showed that the thin filament containing the Leu mutant had a greater activity than the C190A both in the regulated thin filament (i.e. in the presence of Tn ± Ca2+) and in the absence of Tn. In agreement with Fig. 4A, in the absence of Ca2+ the activities were about the same.

DISCUSSION

Our principal finding is that Asp-137 destabilizes a region in the middle of Tm, resulting in a more flexible molecule that is important for modulating the cooperative activation of the thin filament by myosin. We interpret this unstable region in native Tm as a “weak spot” around which the molecule could bend or flex when subjected to a perturbation. Thus, Tm appears to be a non-uniform flexible rod as opposed to a uniform flexible rod. This study therefore proposes that dynamic instability produces flexibility in Tm and furthermore that flexibility and function are linked.

Previous studies have shown that thermal and denaturant-induced local unfolding of 30–60 amino acid residues in the middle of the Tm α-helix takes place prior to the main global transition (28–32). In a preliminary study we reported that Leu substituted for Asp-137 in C190A control, shifted the main transition by +5°, and increased the magnitude of the local unfolding pre-transition (11). Locally unfolded α-helices are necessary to allow accessibility for proteolytic cleavage (33–35).
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![Graph](image_url)

**FIGURE 4. Effect of the Leu mutation on actin-S1 ATPase activity.** A, Ca\(^{2+}\) dependence for fully regulated reconstituted thin filaments. D137L/C190A ( ), C190A ( ). Solution conditions: 3 mM MOPS, 4 mM MgCl\(_2\), 10 mM NaCl, 1 mM EGTA/NTA, pH 7.2. The average S.D. is indicated with the error bars. Hill coefficients and Ca\(^{2+}\) binding constants are shown in the table. B, S1 dependence. D137L/C190A (squares), C190A (circles). Tn plus Ca\(^{2+}\) ( ), Tn minus Ca\(^{2+}\) ( ). Presence of Tm, absence of Tn and Ca\(^{2+}\) ( ). Solutions conditions: [actin] = 2.2 μM; [Tm] = 1.2 μM; Buffer: 30 mM MOPS, 5 mM MgCl\(_2\), 25 mM NaCl, pH 7.2.

Early work with rabbit skeletal Tm showed that Arg-133 is the preferred site of initial cleavage by trypsin and noted that it was close to Asp-137, the only Asp in the hydrophobic ridge of the coiled-coil heptad repeat (25).

We demonstrate here that when Asp-137 is replaced with Leu, the susceptibility of Tm to trypsin cleavage is removed. Another study indicated that V8 protease preferentially cleaves Tm at Glu-131, indicating that the unstable region extends somewhat further to position 131. The site of trypsin cleavage in proteins is the peptide bond C-terminal to Lys and Arg. There are 53 Arg and Lys residues scattered fairly evenly across the 284 residues of Tm, with many located between positions 133 and 190. The lack of limited proteolysis at other regions in the middle of Tm suggests that the earlier observed unfolding pre-transition does not simply involve a single homogeneous cooperative unfolding unit. Rather, the region around Asp-137 preferentially unfolds before other unstable regions in the middle of Tm. Tryptic cleavage at Arg-133 also occurs at 0 °C (25), far from the pre-transition, further indicating that the Asp-137 region is relatively more unstable than the rest of the molecule. At higher temperatures, other N-terminal sites closer to Cys-190 become progressively accessible to trypsin and the rate of digestion increases with temperature and correlates with the temperature dependence of the unfolding in the pre-transition between 35 and 40 °C (30). Regions of instability could result from structural domains that are unfolded or that experience conformational motions resulting in local low amplitude unfolding fluctuations. Local fluctuations in globular proteins have been studied using enzymatic digestion (36), and cooperative unfolding intermediates in globular proteins have been dissected with H-exchange studies (37). Under these conditions (EX2) (36, 38), the observed cleavage rate, \(k_{obs}\), is limited by the fraction of time the molecule is in the cleavable state. Consequently, a large \(k_{obs}\) indicates a high degree of conformational fluctuation at the cleavage site. The observation that Tm is cleaved primarily at Arg-133 and that Tm is protected from cleavage when Asp-137 is replaced by Leu is consistent with both an appreciable amount of fluctuation in the region of Arg-133 compared with other regions and with the idea that Asp-137 is critical for this fluctuation.

For both the Leu mutant and its controls, digestion at Lys-7, resulting in a truncated N terminus, occurs at about the same rate as cleavage at Arg-133. This was shown by MALDI-TOF MS where similar amounts of intact and truncated species were present at short times of trypsin treatment (Fig. 2). This truncated Tm is also seen on SDS gels by a slightly faster mobile species (Fig. 1). In agreement with previous work (25), a C-terminal tryptic fragment at Arg-244 is also observed at later times. Truncation at the ends for the Leu mutant occurs without cleavage in the middle of the molecule at about the same rate as cleavage for the controls. This suggests that truncation appears to occur independently of cleavage for the controls. It is interesting to note that the cleavage at Arg-244 in the C-terminal region did not occur at any of 4 other Lys located closer to the C terminus.

Tm cleavage at Arg-133 is also observed when Tm is bound to actin. However, the rate of cleavage was reduced 10-fold. Thus, actin stabilizes the folded state of Tm or reduces the degree of fluctuation. Because the N-terminal truncation at Lys-7 by trypsin was also inhibited, the ends were also stabilized on actin. This is in agreement with the end-to-end interaction due to overlap involving ~9–11 amino acids (26, 27).

The Leu substitution did not alter the binding of Tm to actin. However, the Leu substitution did significantly increase the Ca\(^{2+}\)-regulated S1-thin filament ATPase and reduced the inhibition by Tm on the S1-actin ATPase (Fig. 4). We interpret this observation in terms of changed equilibria within the three-state model of thin filament regulation (B-C-O) (5). Because the Ca\(^{2+}\) sensitivity is unchanged, the B-C equilibrium does not appear to be affected. In contrast, the increased activity at high Ca\(^{2+}\) and the greater activity in the absence of Tn may reflect a shift in the C-O equilibrium or an increase in the cooperative unit size, \(n\) (39), which is consistent with a decrease in flexibility. In another context, it is interesting to note that an increase in activation by gizzard Tm compared with skeletal Tm has

Z. Grabarek, private communication.
been associated with an increase in n due to stronger end-to-end interactions, which increased communication between Tms (40).

Recent structural studies have suggested that conserved Glu-218 in the hydrophobic a-position could also destabilize the structure (41), particularly because there is a bend in this region. However, no indication of trypsin cleavage near Glu-218 is seen in the absence or presence of actin, even though there are several Lys and Arg nearby. Because the rate of proteolysis of a region is related to the fraction of time that it is unfolded, proteolytic cleavage is a probe for local unfolding fluctuations. Thus, the difference in proteolytic susceptibility reflects a difference in the local dynamics at these two positions.

Brown et al. (24) determined the x-ray structure of a large fragment of Tm containing Asp-137. A cavity in the hydrophobic core was observed between the two heptad repeats bounded by Met-127 and Met-141. Because no significant change in the distance between the helices was observed for this region, this cavity is due to a rotation of the side chains out of the hydrophobic core. An alignment of the backbone in the Leu-207 region with the Asp-137 region provides an insight into the “knob-into-holes”-type packing one would expect if a canonical hydrophobic side chain were present at the 137 position (Fig. 5). As expected, the Leu side chains interact within the hydrophobic core in comparison with the charged acidic side chains that are extended out of the hydrophobic core. The observation of a loss of side chain interactions indicated by the cavity is consistent with decreased stability and increased proteolysis. Our observation of flexibility in Tm at Asp-137 is supported by a recent publication by Straussman et al. (42), noted after this work was completed, that shows that localized bends are produced in the myosin rod coiled-coil where Asp residues are introduced into its hydrophobic ridge.

Asp-137 in muscle tropomyosin is highly conserved across a wide range of organisms from trematodes to humans. Thus, its role in destabilizing the middle of tropomyosin to produce a conformationally flexible region is fundamental to its function. Why would a more flexible, less stable Tm be desirable? Previous data indicate that flexibility is desirable for actin binding (31, 32). Our data suggest that function can be affected by increased flexibility without changing actin binding. Under physiological conditions at the high local myosin concentration, the system may be excessively “turned-on” if Tm is too rigid. A molecule without the required flexibility at this position may not properly modulate the Closed to Open shift associated with activation. We suggest that the correct degree of flexibility is critical to finely tune the regulatory dynamics. There are several single site mutations that are associated with familial hypertrophic cardiomyopathy (6–10). While the Leu mutation is not associated with familial hypertrophic cardiomyopathy, this study provides a basis to understand how some Tm point mutations that are associated with skeletal and cardiac myopathies can modulate Tm dynamic properties to affect regulatory function.

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