A composite approach towards a complete model of the myosin rod

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Abstract

Sarcomeric myosins have the remarkable ability to form regular bipolar thick filaments that, together with actin thin filaments, constitute the fundamental contractile unit of skeletal and cardiac muscle. This has been established for over fifty years and yet a molecular model for the thick filament has not been attained. In part this is due to the lack of a detailed molecular model for the coiled-coil that constitutes the myosin rod. The ability to self-assemble resides in the C-terminal of the section of myosin known as light meromyosin (LMM) which exhibits strong salt dependent aggregation that has inhibited structural studies. Here we evaluate the feasibility of generating a complete model for the myosin rod by combining overlapping structures of five sections of coiled-coil covering 164 amino acid residues which constitute 20% of LMM. Each section contains ~7-9 heptads of myosin. The problem of aggregation was overcome by incorporating the globular folding domains, Gp7 and Xrcc4 which enhance crystallization. The effect of these domains on the stability and conformation of the myosin rod was examined through biophysical studies and overlapping structures. In addition, a computational approach was developed to combine the sections into a contiguous model. The structures were aligned, trimmed to form a contiguous model, and simulated for >700 ns to remove the discontinuities and achieve an equilibrated conformation that represents the native state. This experimental and computational strategy lays the foundation for building a model for the entire myosin rod.

Keywords

Coiled-coil; Protein structure; Molecular dynamics; X-ray crystallization; Cardiac muscle

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Introduction

Cardiac and skeletal muscles contain sets of interdigitated thick and thin filaments. Muscle contraction occurs when the thick filaments, which are built primarily from myosin II, actively slide past the actin-containing thin filaments. Myosin plays both a structural and enzymatic role in this fundamental biological process. The globular N-terminal domains (heads) hydrolyze ATP and interact with actin to generate force, whereas the C-terminal region forms a long α-helix that dimerizes to form a coiled-coil. The coiled-coil is known as the myosin rod. The extended region of myosin can be split in two sections: Subfragment-2 (S2) and light meromyosin (LMM), where these are connected by a flexible section of the coiled-coil that functions as a hinge. In cardiac β-myosin LMM extends from approximately amino acid residues 1140 to 1935.[1] LMM self-assembles to form the backbone of the thick filament at physiological ionic strength. At this time, there is considerable knowledge of the structure and function of the globular motor regions of myosin, but many aspects of the molecular organization of the myosin rods in the thick filament are still unresolved. This has important implications since a significant number of mutations that lead to skeletal and cardiac myopathies are located in the myosin rod.[2-4]

The reason that the structure of the myosin thick filament is not well understood is due to the lack of high resolution structural information for LMM and the complexity of the assembly. Although the myosin rod is predicted to consist of a coiled-coil, which is in principle a simple motif, there are distinct locations where the sequence deviates from a canonical structure.[5] Indeed, there are four conserved locations that contain an additional "skip" residue that disrupts the coiled-coil heptad repeat. These skip residues have been proposed to introduce flexibility into the myosin rod to allow assembly into the thick filament.[6,7] Surrounding these skip residues, the sequence exhibits a 28 amino acid repeat and contains an alternating pattern of positively and negatively charged amino acids, a feature that has been implicated in the staggered interaction between adjacent myosin molecules in the thick filament.[6] In addition, the coiled-coil propensity in the proposed hinge region between S2 and LMM is considerably lower than the sequence signature for a stable coiled-coil. This region is adjacent to the first skip residue and has been proposed to provide an area of flexibility that allows the motor domains swing away from the myosin thick filament to interact with the actin thin filaments.[6,8]

Previous studies on tropomyosin and intermediate filaments have shown that the local pitch of a coiled-coil is profoundly influenced by the amino acid sequence, especially in those regions that deviate from a canonical heptad repeat.[9,10] As such, it is not possible to accurately predict the structure of the myosin rod with the accuracy needed to build a realistic model for the rod and to understand the interactions between adjacent myosin molecules in the thick filament. Clearly there is a need for high resolution structural data.

The sheer size of the myosin rod at approximately 1600 Å in linear dimension, prohibits a direct crystallographic study. Thus, the only feasible approach is to divide the rod into smaller sections that might be more amenable to structural study. Indeed, this is the standard approach for investigating large intractable proteins, but it is more complicated for

fragments of the myosin because the rod exhibits a strong salt-dependent aggregation. A direct result of this property is that fragments of myosin readily form paracrystals at low ionic strength.[11-15] This is due in part to periodic clusters of positively and negatively charged residues that repeat every 28 amino acid residues for the entire length of the myosin rod noted above.[5] In sarcomeric myosins the rod contains 38 of these repeats.[6] The latter property has doubtlessly contributed to the difficulties of crystallizing short sections of the myosin rod that might otherwise appear amenable to structural study. Indeed, until recently there were no structures available for the myosin rod except for the N-terminal 126 amino acid residues of human cardiac β-myosin II S2, which exhibits for most of its length a canonical coiled-coil and has no tendency to aggregate at low ionic strength.[16]

Apart from the problems of self-assembly into paracrystals, a second issue is whether fragments of myosin contain all of the information necessary to fold correctly and yield a soluble protein. The folding of coiled-coils is generally highly cooperative where initiation of folding is often controlled by trigger sequences that have a higher coiled-coil propensity than the bulk of the protein. These sequence signatures usually lie towards the N-terminus of the coiled-coil.[17-22] Removal of these motifs frequently prevents proper folding or loss of dimerization of the resultant fragment. Indeed, a recent paper by Wolny et al., shows that there appears to be a folding signal located in the myosin tail between 1301 and 1330 that leads to high helicity in even short peptides (7 heptads) that contain this sequence. Conversely, five other down-stream peptides that contained only seven heptads of the myosin rod showed limited α-helical content.[23] This problem of stabilizing coiled coils that lack their native trigger sequence has been circumvented for some coiled-coils by incorporation of a short stable coiled-coil, such as that seen in the well-studied transcription factor GCN4.[18,24,25] This strategy could be applied to sections of LMM but is not predicted to eliminate self-assembly into paracrystals at low ionic strength.

The problem of myosin self-assembly has now been solved by using globular fusion proteins in place of GCN4.[26] This strategy was developed for investigating the overlap complex for tropomyosin and components of the yeast spindle pole body and has been recently applied to myosin.[26-28] In those structural studies, the globular domains from human DNA ligase binding protein Xrcc4 and the bacteriophage φ29 scaffolding protein Gp7 were fused to the N-terminus of the targeted coiled-coil domains.[29-31] The resultant fusion proteins were expressed with good solubility in *Escherichia coli* and crystallized readily. Following a similar strategy, the structure of the regions surrounding the four skip residues in human cardiac β-myosin II have been reported which now opens up the possibility of determining the structure of the entire myosin rod.[26] The use of globular domains also solves the problem of the limited stability of short sections of the myosin rod.[23]

In principle, a model for the entire LMM (~800 amino acid residues) could be assembled from the structures of 15-20 overlapping fragments that each span 50-70 amino acid residues. The initial stages of this project are reported here and establishes proof of concept for the approach. This study reports a composite model for residues L1526-L1689 of human cardiac β-myosin generated from five overlapping structures, covering ~24% of LMM. This study also describes the degree of stabilization that can be obtained from a suite of folding domains and their effect on protein expression and crystallization. Molecular dynamic (MD)

simulations of myosin coiled-coil with and without a folding domain provided a means to assess changes in dynamic properties brought about by the fusion. Additionally the fusion structures presented here have a large degree of sequence overlap that provides a basis for evaluating the effects of the fusion protein and crystallographic contacts on the targeted coiled-coil. Lastly, surface analysis shows that the composite model exhibits a substantial hydrophobic surface area which plays a role in the higher order assembly of bipolar thick filaments.

Materials and Methods

All cloning was performed as described previously.[28] Briefly, QuikChange cloning was used to amplify and insert sections of the *MYH7* gene into vectors containing the desired solubilization domain. DNA encoding human cardiac myosin rod was purchased as an image clone from Open Biosystems. The use of QuikChange cloning allowed all constructs to be made without introduction of cloning artifacts, which is critical for maintaining the correct coiled-coil registration between the folding domains and the target gene fragment. The sequences of all constructs were verified over their entire open reading frames.

Construct design

All fusion protein constructs were cloned into a modified pET24D (EMD) plasmid or pKLD37, a modified pET31b plasmid (EMD) by QuikChange cloning.[32-35] A His-tag and an rTEV cleavage recognition site were introduced N-terminally to constructs that contained Gp7, Xrcc4, and GCN4.[36] The Paircoil algorithm was used to predict regions of coiled-coil and their helical registration.[37] A complete description of all constructs is given in Table 1.

Protein expression and purification

Myosin fusion proteins were expressed in an *E. coli* BL21-CodonPlus (DE3)-RIL cell line (Stratagene). Cells were grown in lysogeny broth (LB) medium under an appropriate antibiotic selection at 37 °C with shaking to an A_{600} of ~1.0, then cooled on ice for 15 min; at this point, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and the cells were grown for an additional 16 h at 16 °C before harvesting by centrifugation. Cells were washed with 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.6, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) at a ratio of 1L of buffer per 6L of culture prior to flash freezing in liquid nitrogen and storage at −80 °C.

All protein purification steps were carried out at 4 °C. 10 g of cell were lysed in 100 mL of lysis buffer (50 mM HEPES pH 7.6, 50 mM NaCl, 20 mM imidazole, 0.5 mg/mL lysozyme) with 1 mM PMSF, 50 nM Leupeptin (Peptide International), 70 nM E-65 (Peptide International), 2 nM Aprotinin (ProSpec), and 2 μM AEBSF (Gold BioTechnology) by sonication. Lysate was clarified by centrifugation at 125,000 *g* for 30 min at 4 °C in a Ti-45 rotor. The supernatant was loaded onto a 5 mL Ni-NTA (nickel-nitrilotriacetic acid) column (QIAGEN) by gravity and washed with 25 column volumes of buffer A (25 mM HEPES pH 7.6, 300 mM NaCl, 20 mM imidazole). The column was then washed with an additional 5 column volumes buffer A with 40 mM imidazole. Protein was eluted in four column volumes of buffer A with 200 mM imidazole. 1 mM β-mercaptoethanol was added to all

buffers for purification of constructs containing cysteine residues. The His-tag was removed by incubation with a 1:40 molar ratio of rTEV protease to myosin fusion protein at 4 °C in 25 mM HEPES pH 7.6, 100 mM NaCl, 0.1 mM EDTA, 0.5 mM tris(2-carboxyethyl)phosphine. The NaCl concentration was increased to 300 mM and the cleaved protein was then loaded onto a 2 mL Ni-NTA column equilibrated in buffer A without imidazole. Myosin constructs were eluted in four column volumes of buffer A, and rTEV protease was eluted with buffer A containing 200 mM imidazole. Fusion proteins were concentrated in an Amicon Ultra-15 30 KDa cutoff (Millipore) to between 10 and 25 mg/mL prior to overnight dialysis into storage buffer (10 mM HEPES pH 7.6, 100 mM NaCl). The protein was then flash-frozen in 30 μL droplets in liquid nitrogen and stored at −80 °C.

Crystallization

Crystals of Gp7-1526-1571 were grown at 4 °C by vapor diffusion from a 1:1 mixture of 15 mg/mL protein solution and a polyethylene glycol (PEG) solution consisting of 16% (w/v) PEG 8000, 400 mM malonate pH 7.2, and 100 mM triethanolamine pH 7.5. Rod-shaped crystals grew over the course of several days to final dimensions of 400 μm × 50 μm × 50 μm. All manipulations of crystals prior to freezing were carried out at 4 °C. Crystals were cryo-protected by first being transferred to synthetic mother liquor solution consisting of 18% (w/v) PEG 8000, 400 mM malonate pH 7.2, 100 mM triethanolamine pH 7.5, and 100 mM NaCl followed by stepwise transfer to a final solution of 18% (w/v) PEG 8000, 400 mM malonate pH 7.2, 100 mM triethanolamine pH 7.5, 100 mM NaCl, and 10% (w/v) ethylene glycol. Crystals were flash-frozen by plunging them into liquid nitrogen.

Crystals of reductively methylated[38] Xrcc4-1562-1622 were grown at 4 °C by vapor diffusion from a 1:1 mixture of 13 mg/ mL protein solution and polyethylene glycol (PEG) solution consisting of 23% (w/v) PEG 4000, 500 mM NaCl, 100 mM triethanolamine pH 8.0. Shard-shaped crystals grew over the course of 7 days to an average dimension of 250 μm × 100 μm × 75 μm. All manipulations of the crystals were performed at 4 °C. Crystals were cryo-protected by first being transferred to synthetic mother liquor solution consisting of 23% (w/v) PEG 4000, 500 mM NaCl, 100 mM triethanolamine pH 8.0 followed by stepwise transfer to a final solution of 23% (w/v) PEG 4000, 500 mM NaCl, 100 mM triethanolamine pH 8.0, 12.5% (w/v) ethylene glycol, 250 mM CaCl$_2$. Crystals were flash frozen by being rapidly plunged into liquid nitrogen.

Crystals of reductively methylated[38] Xrcc4-1590-1657 were grown at 4 °C by vapor diffusion from a 1:1 mixture of 15 mg/ mL protein solution and methyl ether polyethylene glycol (MEPEG) solution consisting of 16% (w/v) MEPEG 2000, 250 mM KNO$_3$, 100 mM MOPS pH 7.0. Hexagonal shaped crystals formed over the course of 7 days to an average dimension of 600 μm × 600 μm × 200 μm. Crystals were cryo-protected by transferring to an initial synthetic motherliqour solution consisting of 16% (w/v) MEPEG 2000, 200 mM KNO$_3$, 100 mM MOPS pH 7.0 followed by a stepwise transfer into a final solution consisting of 20% (w/v) MEPEG 2000, 200 mM KNO$_3$ and 12% (w/v) ethylene glycol. Crystals were flash frozen by being rapidly plunged into liquid nitrogen.

Crystals of Xrcc-1631-1692 were grown at 4 °C by vapor diffusion from a 1:1 mixture of 14 mg/ mL protein solution and MEPEG solution consisting of 14% (w/v) MEPEG 5000, 200

mM glycine, 100 mM bistrispropane pH 7.0. Crystals were cryoprotected by transferring to a cryoprotection solution of 20% (w/v) MEPEG 5000, 100 mM NaCl, 200 mM glycine, 100 mM bis-tris propane pH 7.0, followed by a stepwise transfer into a final solution consisting of 20% (w/v) MEPEG 5000, 200 mM glycine, 100 mM bis-tris propane pH 7.0, 12.5% (w/v) ethylene glycol, 200 mM CaCl$_2$. Crystals were then flash frozen by plunging into liquid nitrogen.

Data collection and structure determination

X-ray diffraction data were collected at beam line SBC 19-ID (Advanced Photon Source). The datasets were integrated and scaled using HKL3000.[39,40] X-ray data collection statistics are given in Table 2. The structures of Gp7-1526-1571, Xrcc4-1562-1622, Xrcc4-1590-1657, and Xrcc4-1631-1692 were solved by molecular replacement with Phaser[41,42] using either residues 2 – 52 of Gp7 (1NO4) or residues 1 – 142 of Xrcc4 (1IK9) as search models.[30,31] Following density modification by Parrot, initial models of Xrcc4-1562-1622, Xrcc4-1590-1657, and Xrcc4-1631-1692 were built in Buccaneer.[43,44] Subsequent iterative cycles between manual model building in Coot followed by restrained refinement in Refmac 5.6 were used to generate the penultimate structural coordinates.[45,46] Final refinements for Xrcc4-1590-1657 were performed by TLS and restrained refinement in Refmac 5.6. [42] Two chains were present in the asymmetric unit, and each chain was assigned as an individual TLS group for the entire chain. Final refinements on Xrcc4-1562-1622 and Xrcc4-1631-1692 structures were performed using Phenix Refine.[47] For Xrcc4-1562-1622, four chains were present in the asymmetric unit, with chain A having 3 TLS groups, chain B having 2 TLS groups, chain C having 3 TLS groups, and chain D having 2 TLS groups. Xrcc4-1631-1692 utilized 2 TLS groups for each chain at the junction between the fusion protein and myosin, totaling 4 TLS groups.[46] An initial model of Gp7-1526-1571 was constructed with Phenix AutoBuild and was refined with alternating rounds of manual model building in Coot followed by restrained refinement in Phenix.[47] In the refinement two TLS groups per chain were defined by the junction between the fusion protein and myosin.[48] The eight chains in the asymmetric unit were divided into a total of 16 TLS groups. Refinement statistics are presented in Table 2.

Reconstruction of myosin from structural fragments

The initial structure for the simulations was created in the MODELLER Homology Modeling Package[49-53] starting from the aligned structures for Gp7-1526-1571, Xrcc4-1562-1622, Xrcc4-1590-1657, Xrcc4-1631-1692 and Xrcc4-1551-1609. After alignment, overlapping segments of the structures were trimmed and the orientation of the side chains were energy minimized and the conformation of the trimmed chain ends were optimized to obtain a complete segment consisting of 164 amino acids (MyH7-1526-1689) of the myosin rod surrounding the Skip 3 residue.

All molecular dynamics simulations were carried out using the AMBER v14 MD program.[54-57] The Generalized Born (GB), an implicit solvent approach was chosen because it provides ~100 fold efficiency compared to the explicit solvent when graphical processing units are used,[58-60] and long simulations are required to allow adequate structural relaxations of models constructed based on crystal structures. Specifically, the ff99SB force

field improved with NMR observables (ff99SBnmr) was chosen[61-66] along with the gb7 model.[58] Production gb7 simulations were carried out for a minimum of 500 ns using a 1 fs time step (2 fs was used for the independent runs). Details of the simulations are summarized in Table 3. Langevin dynamics was followed with a collision frequency of 20 ps^{-1} at 300 K. The SHAKE algorithm was applied to bonds with hydrogen atoms with a tolerance of 10^{-5} Å.[67] The non-bonded cutoff was set as 9999 Å, and the maximum distance between atom pairs (rgbmax) for Born radii calculations was kept at 12 Å. Salt was treated implicitly via Debye-Hückel theory and the concentration was set to the physiological concentration of 0.15 M. The collected trajectories were analyzed with AmberTools v15 and the Multiscale Modeling Tools for Structural Biology (MMTSB) package.[57,68,69]

To investigate the effect of the head groups, the isolated Skip 3 region, residues 1551-1603 from the MyH7-1551-1609 structure, was simulated along with fusions with Gp7 and Xrcc4 (Table 3).

In order to examine the convergence of the simulation of the contiguous model, three independent simulations were carried out for a minimum of 520 ns each. The analysis was performed over the combined trajectory of the three simulations where all averages were calculated for the entire pool of conformations. To determine if the simulations had converged the distribution of the clusters among the three individual trajectories was plotted after performing clustering using the *k-clust* algorithm in the MMTSB package.[70] (Figure S1). It was observed that each cluster was sampled in every simulation. Additionally, the averaged D_{COM} and super helical pitch for the individual simulations (Figures S2 and S3) were in qualitative and quantitative agreement with the averages for the aggregate simulations.

CD and fluorescence spectroscopy

Circular dichroism spectra were recorded from 190 to 300 nm in an AVIV model 420 CD spectrophotometer. Protein samples were diluted to an A_{280} of 0.35 in buffer containing 50 mM sodium phosphate pH 7.0, 0.5 mM tris(2-carboxyethyl)phosphine. Thermal melting curves were recorded by monitoring the change in tryptophan emission intensity in a QuantaMaster Model C-60/2000 spectrofluorometer. Fluorescence emissions were recorded from 300 to 500 nm. The excitation wavelength was 290 nm. The temperature was increased by 4 degree increments starting at 4 °C. The sample was equilibrated at each temperature point for 10 minutes before measurement.

Results and Discussion

Fusion proteins do not significantly influence the backbone of the target coiled-coil

Four crystal structures of human cardiac β-myosin LMM are reported here that in combination with the previously determined structure for Skip 3 (Xrcc4-1551-1609 containing ~8 heptads of myosin)[26] encompass residues 1526-1688 (Figure 1). In total this section extends over repeat 25 to the final heptad of repeat 30. These new fragments are defined as Gp7-1526-1571, Xrcc4-1562-1622, Xrcc4-1590-1657, and Xrcc4-1631-1692 that contain ~7, 9, 10, and 8 heptads of coiled-coil respectively. The four new structures overlap

with Xrcc4-1551-1609 on the N- or C-terminal side for a total coverage of LMM of 164 residues (~23 heptads). These structures are N-terminal fusions with either Gp7 or Xrcc4 and were designed such that the overlapping regions of LMM coiled-coil could be used to assemble a larger composite structure. The junctions between the folding domain and myosin were designed to preserve the coiled-coil registry and minimize disruption of inter-chain salt bridges as has been previously described.[27]

As noted earlier, isolated fragments of LMM exhibit a strong dependence salt dependence on solubility and tendency to form paracrystals which confound the ability to crystallize these proteins for high resolution structural studies. This problem was solved by including a globular domain excised from either Xrcc4 or Gp7. These proteins were selected from the protein data bank as small but structurally distinct domains that lead into well ordered dimeric coiled-coils. The intact Xrcc4 is involved in DNA repair and binds to both DNA ligase IV (RCSB accession number 1IK9)[30] and DNA. Here, only the globular domain of the protein that consists of ~140 residues and is composed mostly of β-strands was used, but is referred to as Xrcc4 for simplicity in this context. Likewise, the bacteriophage φ29 scaffolding protein Gp7 consists of a small helical bundle domain that leads into an extended coiled-coil (RCSB accession number 1NO4)[31] where only the first ~50 residues that contain the globular domain were incorporated in these fusions. These are denoted as Gp7-fusions for simplicity also.

Experimentally it was determined that the fusion proteins that exhibited the best characteristics for structural and biophysical studies contained ~8-10 heptads of LMM. Longer fusions did not crystallize well and demonstrated increasing salt-dependent solubility. On average, six constructs were prepared for every successful structural determination.

A major question in assembling structures from fragments derived from fusion proteins is whether the conformation of the resultant pieces is influenced by crystal packing forces or by the interface between the folding domain and the fragment of myosin. In the case of Xrcc4-1562-1622 there are two dimers in the asymmetric unit that have a Cα root mean square deviation of 0.63 Å^2 over 89 α-carbon atoms of the target myosin coiled-coil. This indicates that crystallographic packing does not have a major influence on the resultant structure in this instance. In the same vein, the two fusions to Xrcc4 (Figure 1C and 1D) both overlap between residues 1562-1609 of myosin. This significant overlap is more than the minimum required for assembly into a larger model, but serves as a means to probe the influences of the fusion protein on the target section of coiled-coil. Each of the two Xrcc4-1562-1622 dimers aligns to Xrcc4-1551-1609 with a Cα root mean square deviation of 1.0 Å^2 and 1.2 Å^2 over 76 and 80 α-carbon atoms respectively (Figure 2). This strongly supports the hypothesis that the backbone conformations of the selected fragment of the coiled-coil are not notably perturbed by crystallographic contacts or the fusion to a folding domain.

To further test this hypothesis, MD simulations were performed on the isolated section of MyH7-1551-1602 and fused to either Gp7 or Xrcc4 (Table 3). Structures for the MyH7-1551-1602 segment were extracted from all three simulations to form a pool of

conformations. Hierarchical clustering was performed over the ensemble of conformations for MyH7-1551-1603 using the *k-clust* algorithm in the MMTSB package.[70] Using a Cα root mean square distance (RMSD) of 4.75 Å as the clustering threshold, four clusters were observed. Figure 3A displays distribution of clusters amongst the simulations along with Cα-RMSD with respect to the Xrcc4-1551-1609 crystal structure. The folding domains have a slight stabilizing effect on the conformational ensembles. This is manifested in the average Cα-RMSD. The individual MyH7-1551-1602 simulation has an average Cα-RMSD of 3.8 Å as opposed to 2.4 Å and 3.3 Å from Gp7- and Xrcc4-bound forms. Additionally, the individual MyH7-1551-1602 simulation samples a slightly larger range of conformations; this simulation contains two extra clusters that are not found in the simulation of the Gp7- and Xrcc4-bound constructs. However, those clusters are within 2.7 Å and 2.0 Å Cα-RMSD with respect to the crystal structure (possibly due to the fluctuations at the N-terminal) and have low populations. Cα root mean square fluctuations (Cα-RMSF) exhibit similar trends (Figure 3B). Thus, it can be concluded that the folding domain has a modest stabilizing effect (as supported by the biophysical measurements discussed below), and that the two most dominant conformational ensembles of the coiled-coils are not disturbed.

The conformations sampled in all three simulations are well within comparable RMSD and RMSF values, however this does not necessarily quantify the changes in the degree of coiling. To explicitly quantify the degree of coiling, the distance between center of masses of the two α-helices (D_{COM}) was calculated as a moving average of seven consecutive α-carbon atoms (Figure 3C). All three simulations of the Skip 3 region show similar D_{COM} trends, except for a single heptad repeat at both the C- and N-termini. Hence, the MD simulations support the conjecture that the folding domains and C-terminal truncations do not alter the coiling patterns or the super-helical pitch except for the first and last heptad of the target section of coiled-coil.

Differences in the super-helical pitch at the fusion junction do not propagate into the target coiled-coil

Xrcc4-1562-1622 and Xrcc4-1551-1609 encompass a stretch of LMM that contains the third skip residue, E1582, located between repeat 26 and 27 that breaks the phase of the coiled-coil heptad repeat. The skip residue causes a significant local increase in the super-helical pitch encompassing 17 and 11 residues N- and C-terminal to the insertion. This distorted region of coiled-coil clearly starts at F1565, which places a bulky hydrophobic residue in a core *d* position (Figures 2 and 4). The two Xrcc4 constructs described herein both contain this residue, but in different positions relative to the N-terminal fusion interface. The junction between Xrcc4 and myosin in Xrcc4-1551-1609 is located two full heptads N-terminal to F1565. In contrast, the junction in Xrcc4-1562-1622 is only three residues N-terminal to F1565, or about one turn of an α-helix. As a consequence, the fusion junctions of these two constructs occur at different positions in the coiled-coil registry and include different Xrcc4 residues. The first myosin residue in Xrcc4-1551-1609 is a *d* position occupied by a leucine, while the first myosin residue in Xrcc4-1562-1622 is a glutamine in an *e* position (Figure 4).

The major difference between these two structures occurs in the region surrounding F1565 near the fusion junction in Xrcc4-1562-1622, as indicated by an asterisk in Figures 2 and 4. For approximately two turns of the α-helix, just C-terminal to the folding domain interface, the coiled-coil is more tightly wound in Xrcc4-1562-1622 as compared to that region of myosin in Xrcc4-1551-1609. The coiled-coil of Xrcc4, measured from residues 118 to 153, has a super-helical pitch of about 160 Å, while the skip region of myosin's super-helical pitch is measured at about 900 Å as calculated by the Crick coiled-coil parameterization (CCCP) server.[71] It is unlikely that the structural difference is the result of crystallographic packing, given that conformational variability between crystal structures is typically observed in loops and surface residues, and not in packing residues such as those in the coiled-coil.[72] Rather, this distortion appears to be the result of a mismatch of super-helical pitch between Xrcc4 and myosin. Except for the first five residues adjacent to the fusion protein the target myosin coiled-coils are highly similar. Fundamentally this implies that the structural influence of the folding domain does not propagate significantly beyond the first heptad of the target coiled-coil.

Xrcc4-1551-1609 is the highest resolution structure for that section of myosin coiled-coil and hence used in the construction of the composite model below (Figure 1C and F). The junction between the Xrcc4 folding domain and the target myosin coiled has a smooth transition, where the coiled-coil parameters are well matched. Both Xrcc4-1551-1609 and Xrcc4-1562-1622 exhibit stable coiled-coil C-termini with no significant differences over regions of overlap.

Regions of stable coiled-coil interface should be selected for C-terminal truncations

The most N-terminal segment described in this study, Gp7-1526-1571, contains myosin residues spanning repeat 25 and half of repeat 26 and also includes F1565. Unlike in the previous two Xrcc4 constructs, F1565 is located six residues from the C-terminus of the coiled-coil. Interestingly, the last two heptads of this fusion protein splay apart forming an antiparallel four-helix bundle between crystallographically related dimers (Figure 5). Given the redundancy of structural information for this section of the coiled-coil it is safe to assume that this arrangement is an artifact of crystal packing and was excluded from consideration in the composite model. This tetramerization probably arises because of a weak dimerization interface near the C-terminus of the coiled-coil that is readily counterbalanced by crystallographic packing forces (Figure 5). Interestingly, the coiled-coil prediction for this region is less than 100% in the region leading up to F1565[73] which suggests that a minimum of one heptad of canonical coiled-coil should be included at the C-terminus to avoid formation of antiparallel four helix bundles. The observation that truncated fragments of coiled-coils can come apart at their ends raises the question to what extent the folding domains stabilize these segments of coiled-coil. This was addressed experimentally by determining the folding and unfolding characteristics of a short section of myosin fused to three different folding domains. The same question was also examined earlier in the MD studies.

Fusion proteins modulated the level of expression and increased the total α-helical content of the chimera

Fusions were constructed between the short coiled-coil of GCN4, Gp7, or Xrcc4 and a region of human cardiac myosin, that encompasses the second skip residue (MyH7 residues 1361 to 1406). The fusions were compared to a construct that did not include any folding domain. A mutant form of the GCN4 leucine zipper that was designed to have greater coiled-coil stability (GCN4-pMSE hereafter abbreviated to GCN4) was used as a comparison to two other folding domains.[74] The particular region of myosin was selected because it contains a tryptophan residue enabling fluorescence-based measurements. All MyH7 fusions were purified by Ni-NTA chromatography under identical conditions and the amount of soluble protein was compared using relative band intensity on a Coomassie-stained SDS-PAGE gel. All of the constructs yielded a substantial quantity of soluble protein, where the Xrcc4 fusion yielded the greatest mass of soluble material (Figure 6). Interestingly, although the native coiled-coil protein was soluble without a folding domain, the expression levels do not convey the dramatic differences in biophysical behavior exhibited by these constructs. While MyH7-1361-1406 appeared folded, as indicated by a single sharp peak in gel filtration (data not shown), it had low α-helical content as measured by circular dichroism. The mean residue molar ellipticity was -12733 deg.cm^2.dmol^{-1} at 222 nm which was much lower than a value of ~33,000 expected for a protein that is predicted to be entirely α-helical.[75] This is consistent with the observation by Wolny et al., that many isolated short sections of the myosin rod exhibit low α-helical content.[23] The addition of the folding domain greatly increases the mean residue molar ellipticity, as listed in Table 4, indicating that the stability conferred by the folding domain propagates into the target coiled-coil.

Fusion proteins enhance thermal stability

All fusions greatly enhanced the thermal stability of the target coiled-coil as shown by tryptophan autofluorescence (Figure 7). The control construct lacking an N-terminal fusion had an emission maximum of 355 nm indicating the tryptophan residue is solvent exposed and did not undergo a cooperative unfolding transition in temperature scanning measurements. The emission maximum of the fusion proteins were blue shifted to 342 nm (Gp7) and 340 (GCN4) due to increased shielding from solvent. GCN4 conferred the greatest enhancement of thermal stability. The analysis of Xrcc4 constructs, based on autofluorescence, is complicated by multiple tryptophan moieties and was therefore not included. The CD (Figure 7) and autofluorescence data taken together demonstrate that the fusions increase the total α-helical content and thermal stability of the target coiled-coil suggesting that these constructs are more suitable for biophysical analysis. This was reflected in the crystallization properties of these proteins.

The fusion protein facilitates crystallization

Constructs were evaluated for crystallization in a 144 condition sparse matrix screen at 4°C and 25°C that samples a range of polyethylene glycol, hexylene glycol, and salt, based conditions. The screen was prepared in-house. No crystals have been observed for MyH7-1361-1406 alone, while the Xrcc4, GCN4, and Gp7 fusions readily crystallized, but

even here among the fusions there were significant differences. GCN4 yielded visually stunning crystals, but these exhibited highly anisotropic diffraction. Conversely, the inclusion of a structurally more complex motif such as Gp7 yielded more ordered crystals that were readily amenable to structural analysis. In general, Gp7 fusions crystallize under a wider range of conditions than Xrcc4 fusions. The increased thermal stability of the fusion relative to the target coiled-coil coupled with a tendency for crystallization provide strong support for the use of these folding domains in the context of myosin.

Assembly of structural fragments into a contiguous model

There is considerable structural overlap between the five structures shown in Figure 1. However, a model built by simple alignment represents a crude approximation considering that overlapping regions exhibit slightly different conformations (Cα root mean square deviation of < 1 Å). Although previous studies that focus on modeling canonical coiled-coils have been reported, modeling the myosin rod based on those studies is unrealistic due to the presence the skip residues that lead to deviations from an ideal coiled-coil[76]. Our studies of the skip residues have shown that the deviation from an ideal coiled-coils surrounding a skip residue extends over ~four heptads where the distortions is dependent on the surrounding amino acid sequence.[26] Due to the irregularities in the structure a model based on a typical canonical coiled-coil is incapable of defining the structure of the myosin rod[76]. Consequently a more systematic approach described here was developed to assemble a contiguous structure from the individual structures with the homology modeling program MODELLER.[52]

The initial model from this procedure was simulated through molecular dynamics to remove steric overlaps and allow adequate structural relaxations through three separate simulations. The trajectory length for each simulation is listed in Table 3. MD simulations of the composite model yielded a conformational ensemble rather than a single conformation and thus provide information on the flexibility of the molecule. This was derived from the sum of three independent trajectories (summing up to 2.2 μs).

Hierarchical clustering was performed on the ensemble of structures generated from the simulation to distinguish major conformers (Figure 8). The RMSD cut off for clustering was selected after trying several values varying from 6 Å to 10 Å. Within that range, 8 Å was found to yield the most robust clustering where this best reflected the diversity of the conformations sampled by the simulations. Six clusters were formed when the ensembles were sorted with this Cα–RMSD threshold (Figure 8G). The representative structure of a given cluster was taken as that which had the lowest overall Cα–RMSD to the other members of the cluster. A comparison of the six representative structures, one from each cluster, revealed that the major difference between the ensembles is the degree of supercoiling. At one extreme the Cα–RMSD increases to 10.7 Å between the 4th cluster (shown yellow) relative to the initial model, where the structure is less tightly wound and more flexuous than the initial model. The slight bend towards C-terminal is expected since the structure becomes more flexuous due to length. This particular cluster was only sampled in 5.5% of the overall population.

The simulated composite model for the MyH7-1526-1689 section of the myosin rod was also compared to the simulation of MyH7-1551-1602 extracted from the crystal structure of Xrcc4-1551-1609. This analysis probed how the increased length of the composite model alters the structures relative to simulations of smaller sections. It is important to emphasize that the conformational space visited in each simulation does not change between the simulations of the smaller section and the composite model. Clusters obtained from the composite model simulation remain within 2.1 Å Cα–RMSD of the crystal structure of Xrcc4-1551-1609. The composite model is expected to be flexuous due to its length. MyH7-1526-1689 exhibits higher Cα-RMSF than the MyH7-1551-1603 segment (Figure 8), which is typically in the range of 3-4 Å for the composite model and ~2 Å for Xrcc4-1551-1602 simulation. The highest degree of RMSF is observed between residues 1582-1640, following the skip residue. This prediction from the simulations is consistent with the observed structure for Xrcc4-1590-1657 which shows that the α-helices separate between residues 1608-1615 consistent with a lower predicted coiled-coil propensity.

Since the clustering results pointed to a difference in the degree of coiling among the representative structures, the super-helical pitch of the myosin rod was calculated over the full simulation. Super helical pitch was calculated based on a sliding window of 7 (black) or 14 (red) amino acids. As expected, there is an increased super-helical pitch around the skip residue, extending 20 amino acids upstream from the skip location where the computed super-helical pitch averages over 1300 Å as compared to the value of 170 Å for other regions (Figure 8C) for both approximations (single heptad vs 2-heptads). Both MyH7-1551-1602 and MyH7-1526-1689 follow the same qualitative trend (data not shown). These data taken together strongly support the feasibility of assembling a complete model for LMM and illustrate the difficulties associated with building a model based on a canonical coiled-coil.

The average length of each heptad repeat, or local pitch, was calculated over the trajectory (Figure 8D), to further emphasize the local disruption due to the skip residue. While the local pitch averages to 10 Å for most of the coiled-coil, it rises up to 12.5 Å around the skip residue. D_{COM} (See Methods for details) also shows increased distance between the centers of masses of the two helices around the skip residue and provides further evidence that the thick filament is less tightly wound around the skip residue.

Prediction software, such as that provided by servers such as COILS[77] and MARCOIL,[78] are commonly used to provide an estimate of how well a sequence matches a canonical coiled-coil. While these are outstanding at predicting potential coiled-coils they do not provide direct evidence of the structure adopted by a polypeptide chain in those regions where the sequence deviates from a canonical heptad pattern. There is also considerable discrepancy between predictions for those segments that do not exhibit a standard pattern. As shown in Figure 8E, the predictions from COILS and MARCOIL are not only inconsistent with each other but do not predict the observed structural features seen in our structures and simulations. MARCOIL predicts that the entire region should be coiled-coil with over 99% propensity whereas COILS predicts low coiled-coil propensity around residues 1558 and 1590-1613 region. This comparison demonstrates that the thick filament cannot be modeled using sequence information alone or built on a canonical coiled-coil; the

presence of the skip residues disrupts the expected pattern such that structural information is required to accurately model these regions.

Finally, the interactions within the coiled-coil in the simulated composite model were examined. The electrostatic interactions were analyzed by calculating the average distances between the center of mass of the nitrogen atoms of arginine (NH1, NH2) and lysine (NZ) and the center of mass of oxygen atoms of glutamate (OE1, OE2) and aspartate (OD1, OD2). The distances are averaged over an ensemble of conformations sampled by three independent simulations rather than a static model. Residues with atoms that lie within 4.5 Å distance were selected as interacting pairs. Only a few electrostatic interactions were predicted to be occur between the two helices; those pairs are E1536-K1537, K1579-D1580 and R1604-E1608 (Figure 9A and Table S1). It is important to note that the charged interactions predicted do not follow the well known *e-g'* interactions observed in typical parallel coiled-coils, where *e* type residue on one chain interacts with the preceding *g'* type residue from the other chain.[79] None of the pairs identified in this study match the *e-g'* category. There are however a few ionic interactions between residues in the same chain, notably between the 1602D-1606R and 1615K-1619E pairs, that might contribute to the stability of the assembly (Figure 9B). No repulsive interactions were observed in this 164 amino acid segment of the myosin rod in the simulations.

The stability of the coiled-coil is expected to be driven by the hydrophobic effect. The hydrophobic interactions between two helices of the coiled-coil were evaluated from the average distances between the center of masses of each hydrophobic side-chain (Figure 9C and Table S2 for a full list of interactions identified). The distances were averaged over the ensemble of conformations from three independent trajectories. All hydrophobic pairs whose center of mass of the side chains fall within 5 Å were selected and shown in orange Figure 9D, where these interactions are strictly in the interface between the two α-helices. Almost all interactions can be classified as contacts between *a–d'* or *a–g'* residues.

Unlike most typical soluble globular protein structures, the myosin coiled-coil exhibits a large number of hydrophobic side chains on the surface. There are numerous solvent exposed hydrophobic residues as indicated by the solvent accessible surface area (SASA, $Å^2$) for each hydrophobic side chain (Figure 9A) including, L1526, M1538, L1559, L1563, L1591, L1612, I1627, M1635, L1649, I1655, I1673, and L1680. These all have SASA values over 90 $Å^2$ (Figure 9E and Table S3). The composite model contains 132 hydrophobic residues, of which 24 are almost completely solvent exposed. This solvent accessibility is by far higher than it is expected for hydrophobic residues in α-helices; usually hydrophobic amino acids are only 1% to 5% accessible, except for Trp and Tyr which are 10% accessible.[80] We hypothesize that these residues are important for assembly of the thick filament beyond the simple ionic interactions[81] and will be important in future efforts to construction higher ordered structures. Interestingly, in most instances the hydrophobic character of these positions in the myosin rod is highly conserved across sarcomeric myosins.

Distribution of cardiomyopathy mutations

More than eighteen mutations that lead to cardio or skeletal myopathies have been identified in the segment of myosin modeled here.[3,82] The location of these residues is shown in Figure 10, which reveals a broad distribution of sites. Of these, *in vivo* and *in vitro* measurements have only been reported for the effect of the E1555K substitution.[23] These show reduced incorporation of the mutant protein into the sarcomere and decreased helicity in the coiled-coil which, together with studies of mutations outside the segment investigated here, suggest that mutations that reduce the helix stability lead to lower incorporation into the sarcomere. As shown on the model, E1555 is a surface residue that does not appear to be involved in any inter or intramolecular ionic interactions in the wild-type structure. Thus, the loss of stability cannot be attributed to the loss of a stabilizing interaction, but rather must be due to gain of a destabilizing contact within the coiled-coil. The reduced incorporation into the thick filament could be due to either defects in interactions between myosin rods or by reduced stability of the protein itself. Resolution of this question can now be approached through simulation of the mutant structure and through structural determination of segments that carry mutations.

It is noteworthy that there are a considerable number of mutations to proline that lead to skeletal myopathies.[82,83] *In vitro* studies of two mutations outside the region studied here (R1500P and L1706P) suggest that prolines can be introduced into the myosin rod and yet still lead to protein that is incorporated into thick filaments even though the assembly appears is less stable[83]. It is expected that introduction of a proline into a helical backbone will lead to loss of hydrogen bonding opportunities. However, proline residues are occasionally found in α-helices where they lead to a pronounced bend in their path. For example, there is a proline found in α-helix 2 of thioredoxin from *E. coli* (PDB accession code 2TRX) that leads to a prominent kink in the helix that alters the hydrogen bonding pattern surrounding the proline. This results in an increased rise per residue and loss of an additional hydrogen bond beyond that caused by the proline itself.[84] There are at least eight examples of proline residues in dimeric or trimeric coiled-coils[85] where these also lead to over-winding of the alpha helices.[85] Thus, it is difficult to predict whether proline mutations in myosin lead to alterations in the stability of the coiled-coil itself or to changes in the manner the myosin rods interact with each other in the thick filament. Here again, the computational and structural approach outlined here is well suited to investigating the biophysical consequences of these mutations.

Conclusions

This study establishes a methodology for determining the complete structure of the myosin rod. It shows that the use of fusion proteins allows restricted segments of the myosin rod to be expressed and purified which allows structural and biophysical characterization. It also demonstrates that the fusion domains themselves introduce only a small perturbation in the structure that is readily eliminated by structural determination of overlapping fragments. The study also shows that the fragments can be assembled into composite models through molecular dynamics simulations. The five fragments studied here contained a total of 292 residues of myosin which yielded a contiguous model of 164 residue fragment. This

suggests that a complete model for LMM can be obtained by implementing a strategy that includes duplicate structures for every residue in the coiled-coil. The results thus far show a surprising number of exposed hydrophobic side chains which suggests they may play a role in the assembly of myosin rods into the thick filament. The methodology established here will facilitate construction of a model for the entire myosin rod, and lays the ground work for assembling the rods into a model for the thick filament. This study also creates a framework for understanding the biophysical consequences of mutations in myosin that lead to cardio and skeletal myopathies. These studies are in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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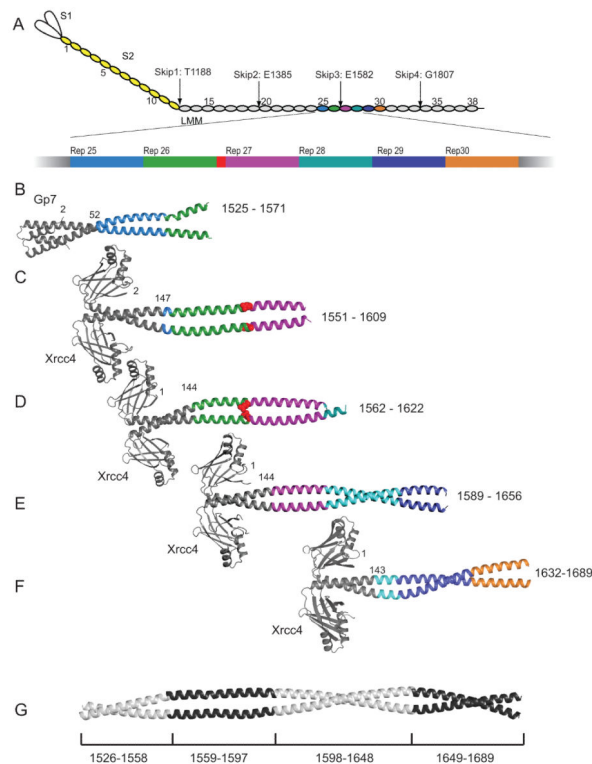
Figure 1.
Structures of myosin fusion proteins and preliminary composite model. (**A**) A representation
of myosin in which each 28 amino acid repeat of the C-terminal coiled-coil is an oval. S1,
S2, and LMM are colored white, yellow and grey respectively. The numbering is shown for
every fifth repeat and the positions of the skip residues are indicated. Repeats 25 through 30
are colored differently and the third skip residue, E1582, is shown in red. The fusion
proteins are colored in grey while the myosin repeats are colored as in panel A for (**B**) Gp7-
L1526-E1571, (**C**) Xrcc4-L1551-N1609, (**D**) Xrcc4-Q1562-L1622. (**E**) Xrcc4-H1590-
L1657, (**F**) Xrcc4-A1632-R1689 and (**G**) A simple composite model for L1526-R1689 of
human cardiac β-myosin. This was assembled from four of the five overlapping structures
taken from Gp7-L1526-E1571, Xrcc4-L1551-N1609, Xrcc4-H1590-L1657, and Xrcc4-
A1632-R1689. The residues incorporated from each structure are listed below. The
coordinates for Xrcc4-L1551-N1609 were taken from the RCSB with accession 4XA4.
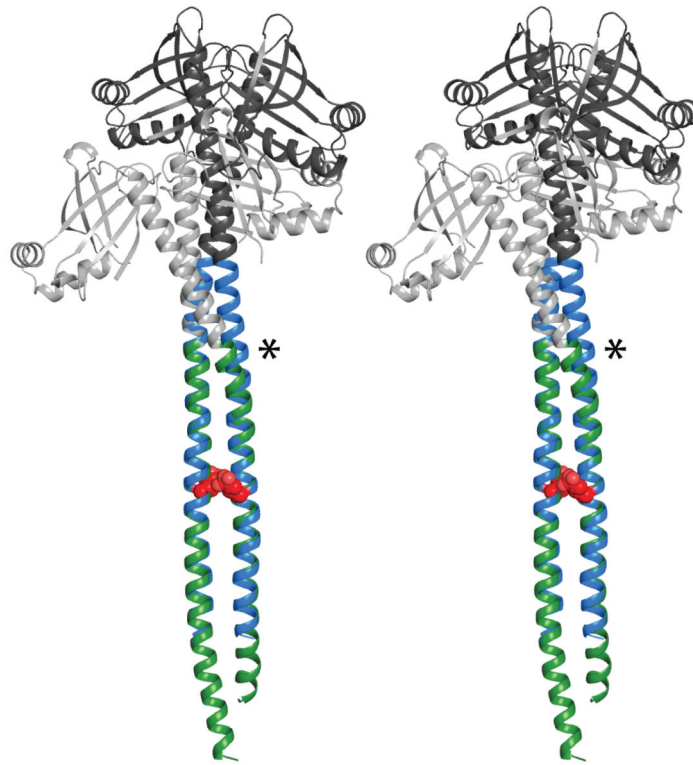Figures 1, 2, 3, 4, 5, 8, 9, and 10 were prepared in part with Pymol (http://www.pymol.org/).

Figure 2.
Stereo view of a structural alignment between Xrcc4-1551-1609 and Xrcc4-1562-1622. For Xrcc4-1551-1609 the Xrcc4 portion is colored in black and 1551-1609 of myosin is in blue. For Xrcc4-1562-1622 the Xrcc4 portion is colored in grey and 1562-1622 of myosin is in green. The structures are represented in cartoon and the skip residue is shown in red spheres. The primary point of divergence of the structures in the target coiled-coil is indicated with an asterisk.
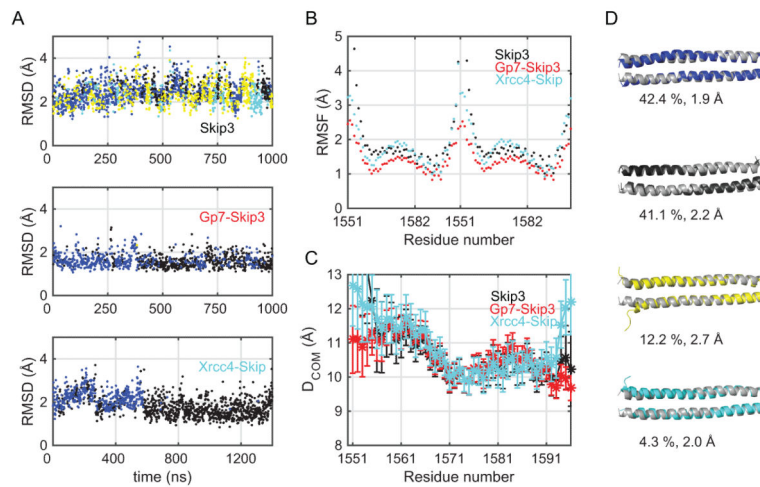
Figure 3.
Analysis of the molecular dynamics simulations for Skip 3 in the absence and presence of a fusion partner. (**A**) Clustering of the Skip 3 region (MyH7-1551-1602) conformations extracted from Skip 3 alone, Gp7-Skip 3 and Xrcc4-Skip 3 simulations, is presented in three individual panels along with the Cα-RMSD with respect to the Xrcc4-1551-1609 crystal structure. Hierarchical clustering was carried out with the *k-clust* protocol in MMTSB using a Cα-RMSD of 4.75 Å as the similarity measure. Different colors represent different clusters. (**B**) Root Mean Square Fluctuations (RMSF) based on the Cα-atoms (Black-isolated Skip 3 simulation, Red: Gp7-Skip 3, Turquoise: Xrcc4-Skip 3). (**C**) D_{COM} trend for Skip 3 (Black), Gp7-Skip 3 (red) and Xrcc4-Skip 3 (turquoise) simulations. (**D**) Representative members from each cluster are shown along with their overall population percentages and Cα-RMSD with respect to the Xrcc4-1551-1609 crystal structure.
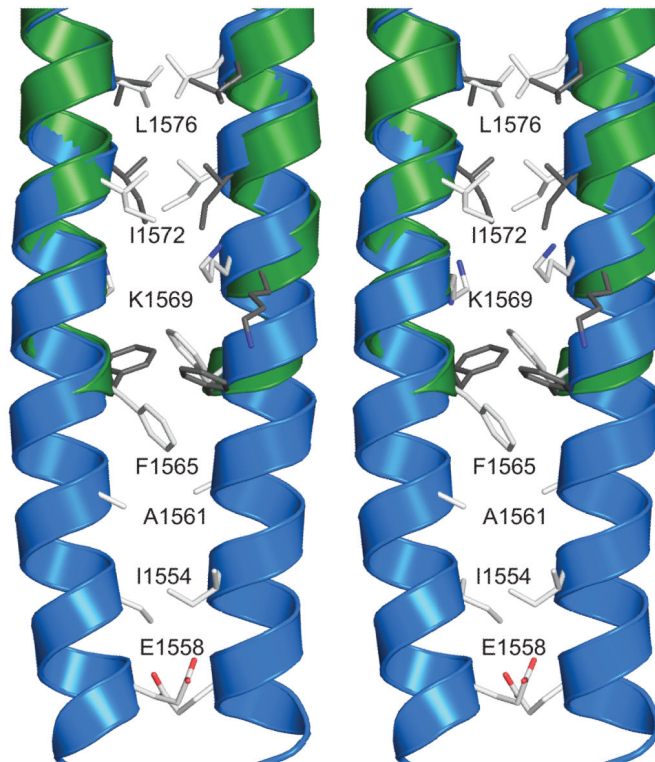
Figure 4.
Stereo representation of the coiled coil centered on F1565 in the Xrcc4-1551-1609 and Xrcc4-1562-1622 fusion proteins. Xrcc4-1551-1609 is represented with blue cartoon helices and white stick side chains. Xrcc4-1562-1622 is represented with green cartoon helices and dark grey side chains. Only the side chains of residues along the interface are displayed. Residues from the Xrcc4 folding domain are not shown.
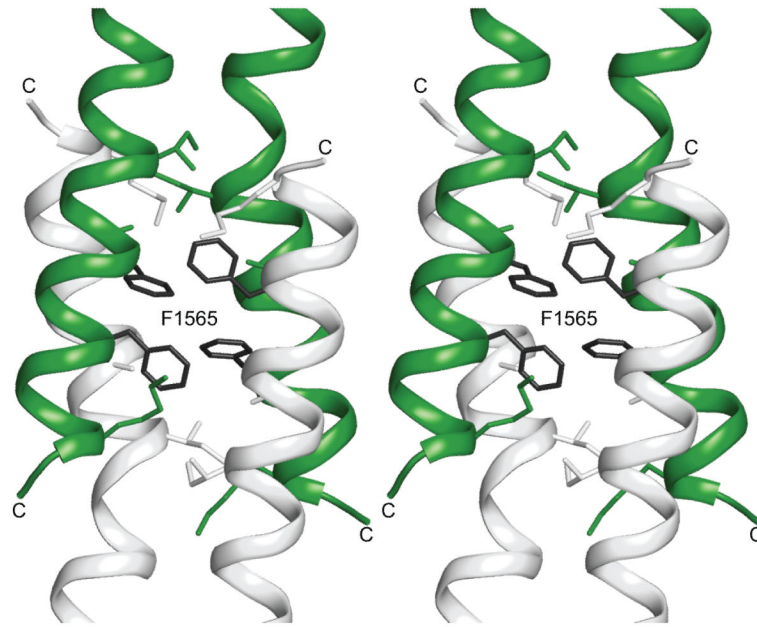
Figure 5.
Stereo diagram showing the antiparallel four helix bundle formed by Gp7-1526-1571. One dimer is colored in green while the symmetry related dimer is colored in white. The clustering of F1565 in all four chains is critical to the formation of the antiparallel helix bundle. The C-termini of the polypeptide chains are indicated.
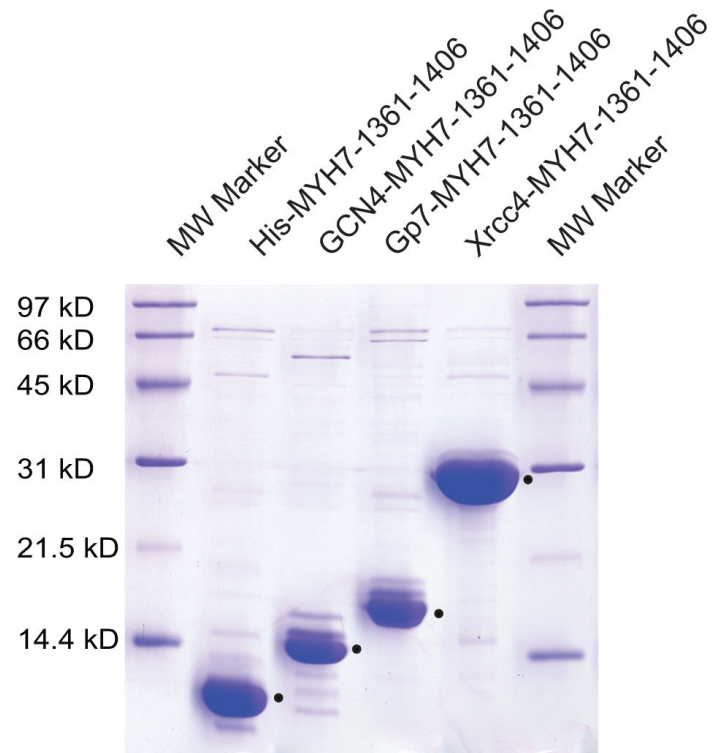
Figure 6.
15% SDS-PAGE gel demonstrating soluble expression of MyH7 constructs. (A) All lanes contain 1.25 μL of a Ni-NTA purified MyH7 construct (equivalent of 0.625 μg of cells). Bands corresponding to MyH7 are marked with a dot to the right of the band. All MyH7 constructs show soluble overexpression, including fusion-less MyH7, and only minimal changes in soluble expression are noted with different fusions. Xrcc4-MyH7 shows the highest soluble expression level.
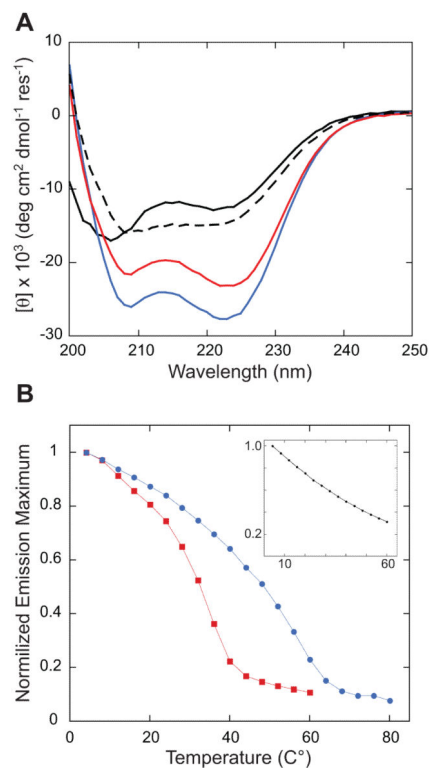
Figure 7.
(A) Circular dichroism spectra of MYH7-1361-1406 (black) and Gp7 (Red), GCN4 (blue), Xrcc4 (black dashed line) fusions to MYH7-1361-1406. (B) Temperature scanning autofluorescence emission maximum for each construct is plotted versus temperature. Gp7 (Red ▪) and GCN4 (Blue ●) fusions to MYH7-1361-1406 show temperature dependent transition. MYH7-1361-1406 (Black ●) without fusion is shown in the inset and does not display a cooperative temperature depend change in tryptophan fluorescence. A line connecting the measured data has been added to each trace as a guide.
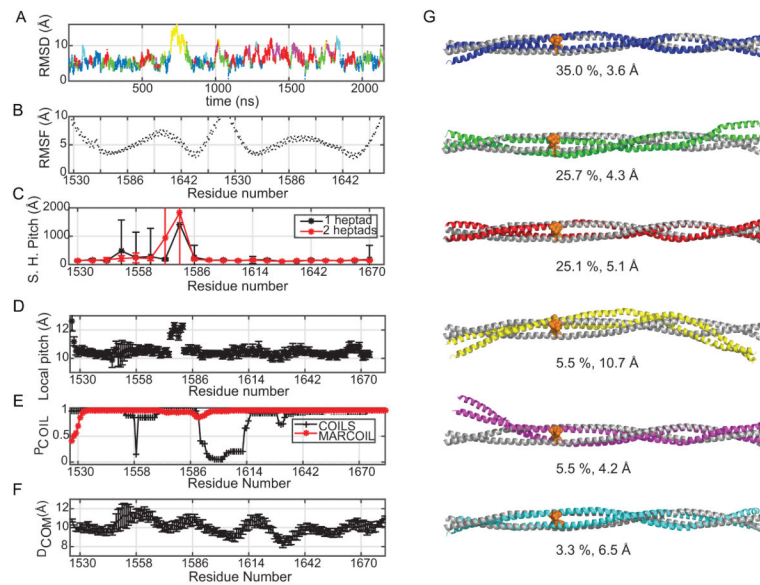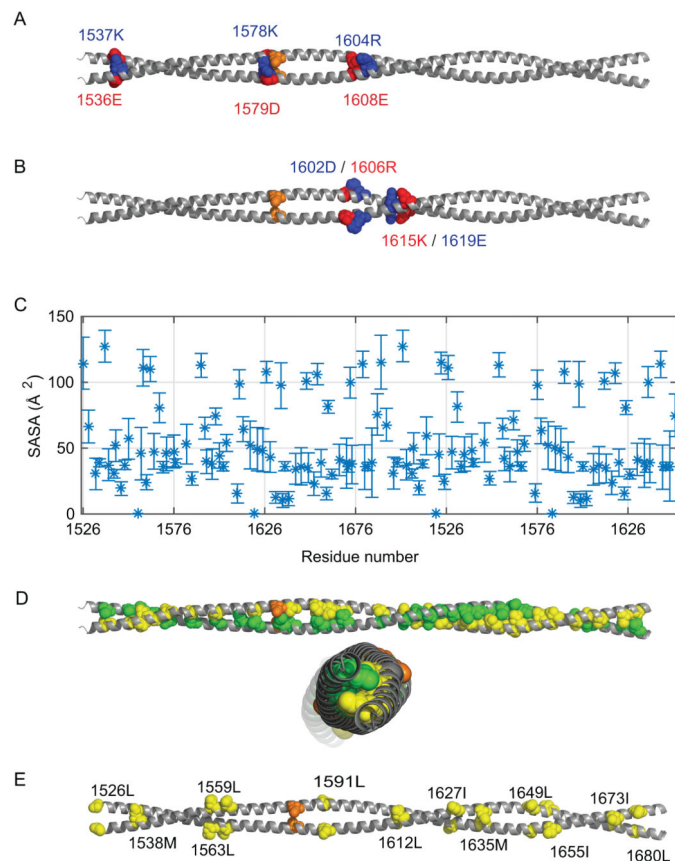
Figure 8.
Molecular dynamics analysis of the MyH7-1526-1689 segment of the myosin rod. (**A**) Cα-RMSD with respect to the initial model that was assembled directly from the crystal structures. Color coding represent different clusters obtained through hierarchical clustering using the *k-clust* protocol in MMTSB. The Cα-RMSD cut off for clustering was set to 8 Å which gives the most robust clusters. (**B**) Root Mean Square Fluctuations (RMSF) based on the Cα-atoms. (**C**) Estimated super-helical pitch (Å) trend over each heptad-repeat (Skip 3 residue: E1582) based on a single heptad (black) and two heptad repeats (red). The values shown are calculated through averaging over conformations from all 3 trajectories. (**D**) The length of each heptad repeat calculated as a moving average to reveal local fluctuations more closely (**E**) Coiled-coil propensities are calculated with a 28 residue sliding window using COILS (red) and MARCOIL (black) servers using only the sequence information (**F**) D_{COM} for the composite model calculated through averaging over conformations from all 3 trajectories. (**G**) Representative members from each cluster are shown along with their overall population percentages and Cα-RMSD with respect to the initial model for MyH7-1526-1689.

Figure 9.
Analysis of the charged interactions and exposed hydrophobic surface of the composite model for MyH7-1526-1689. (**A**) Charged interactions between two helices evaluated by the average distances between center of mass of nitrogen atoms (NH1, NH2) of Arginine, nitrogen atom (NZ) of Lysine and center of mass of oxygen atoms from Glutamate (OE1, OE2) and Aspartate (OD1, OD2). The pairs of residues within 4.5 Å distance are shown (Table S1). The Skip 3 residue is shown in orange. Positively charged amino acids are displayed in blue whereas negatively charged amino acids are displayed in red. (**B**) Similarly, charged interactions within each chain are shown in sphere representation. (**C**) Solvent accessible surface area (SASA) (Å^2) of the hydrophobic amino acid residues in the composite model (Table S2). (**D**) Side and top view of MyH7-1526-1689 are displayed with the predicted hydrophobic interactions. The hydrophobic interactions within the rod were evaluated from the average minimum distances between the hydrophobic side-chains. The pairs of residues that are closer than 5 Å are shown (Table S3). To distinguish residues from different helices, the predicted residues are colored in yellow and green for different helices. (**E**) Hydrophobic residues that have a SASA over 90 Å^2 are shown on the composite model in sphere representation.

Q1541P E1555K   E1573K R1588P T1599P   E1619K   R1634C   D1652Y A1663P   R1689C
                              L1591P A1603P   A1636P L1646P
                                      R1608P   A1637T R1662P
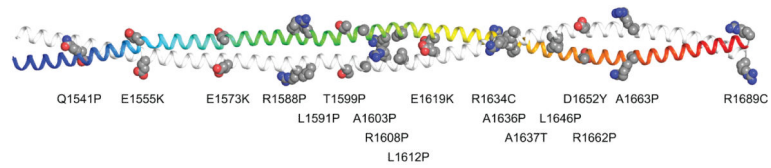   L1612P

Figure 10.
Disposition of mutations that lead to cardio or skeletal myopathies in MyH71526-1689. The mutations themselves are widely distributed along the length of the coiled-coil and occur at all heptad positions.

Table 1

Fusion constructs*a*.

MyH7-1361-1406
MSYYHHHHHHDYDIPTSENLYFQG<u>LSKANSEVAQWRTKYETDAIQRTEELEEAKKKLAQRLQEAEEAVEA</u>

GCN4-MyH7-1361-1406
MGSSHHHHHHHHDYDIPTSENLYFQGASMSVKELEDKVEEL<u>LSKNYHLENEVARLKKLLSKANSEVAQWRTK</u>
<u>YETDAIQRTEELEEAKKKLAQRLQEAEEAVEAVNA</u>

Gp7-MyH7-1361-1406
MSYYHHHHHHDYDIPTSENLYFQGGSGPLKPEEHEDILNKLLDPELAQSERTEALQQLRVNYGSFVSEYNDL
TKS<u>LSKANSEVAQWRTKYETDAIQRTEELEEAKKKLAQRLQEAEEAVEAVNA</u>

Xrcc4-MyH7-1361-1406
MSYYHHHHHHDYDIPTSENLYFQGGSGERKISRIHLVSEPSITHFLQVSWEKTLESGFVITLTDGHSAWTGT
VSESEISQEADDMAMEKGKYVGELRKALLSGAGPADVYTFNFSKESCYFFFEKNLKDVSFRLGSFNLEKVEN
PAEVIRELICYCLDTTAENQAKNEHLQKENERLQRV<u>LSKANSEVAQWRTKYETDAIQRTEELEEAKKKLAQR</u>
<u>LQEAEEAVEAVNA</u>

Gp7-MyH7- MyH7-1526-1571
MSHHHHHHHHDYDIPTSENLYFQGGSGPLKPEEHEDILNKLLDPELAQSERTEALQQLRVNYGSFVSEYNDL
TKSHEK<u>LEKVRKQLEAEKMELQSALEEAEASLEHEEGKILRAQLEFNQIKAE</u>

Xrcc4-MyH7-1562-1622
MSYYHHHHHHDYDIPTSENLYFQGGSGERKISRIHLVSEPSITHFLQVSWEKTLESGFVITLTDGHSAWTGT
VSESEISQEADDMAMEKGKYVGELRKALLSGAGPADVYTFNFSKESCYFFFEKNLKDVSFRLGSFNLEKVEN
PAEVIRELICYCLDTTAENQAKNEHL<u>QLEFNQIKAEIERKLAEKDEEMEQAKRNHLRVVDSLQTSLDAETRS</u>
<u>RNEALRVKKKME</u>**GDL***b*

Xrcc4-MyH7-1590-1657
MSYYHHHHHHDYDIPTSENLYFQGGSGERKISRIHLVSEPSITHFLQVSWEKTLESGFVITLTDGHSAWTGT
VSESEISQEADDMAMEKGKYVGELRKALLSGAGPADVYTFNFSKESCYFFFEKNLKDVSFRLGSFNLEKVEN
PAEVIRELICYCLDTTAENQAKNEH<u>HLRVVDSLQTSLDAETRSRNEALRVKKKMEGDLNEMEIQLSHANRMA</u>
<u>AEAQKQVKSLQSLLKDTQIQL</u>

Xrcc4-MyH7-1631-1692
MSYYHHHHHHDYDIPTSENLYFQGGSGERKISRIHLVSEPSITHFLQVSWEKTLESGFVITLTDGHSAWTGT
VSESEISQEADDMAMEKGKYVGELRKALLSGAGPADVYTFNFSKESCYFFFEKNLKDVSFRLGSFNLEKVEN
PAEVIRELICYCLDTTAENQAKNE<u>HANRMAAEAQKQVKSLQSLLKDTQIQLDDAVRANDDLKENIAIVERRN</u>
<u>NLLQAELEELR</u>**AVV***b*

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*a*The myosin segment is underlined.

*b*The residues highlighted in bold were not observed in the crystal lattice though were present in the protein.

Table 2

Crystallographic data collection and refinement statistics

Data Collection	Gp7-1526-1571	Xrcc-1562-1622	Xrcc4-1590-1657	Xrcc4-1631-1692
Space group	P1	P2$_1$	C2	C2
Cell dimensions				
a, b, c (Å)	56.1, 64.5, 70.5	83.1, 57.3, 112.1	223.6, 84.0, 39.4	200, 44.4, 74.7
α,β,γ (°)	70.5, 77.5, 74.2	β = 99.9	β=92.8	β =108.2
Wavelength (Å)	0.9791	0.9791	0.97924	0.9791
Resolution (Å)[a]	50-2.1 (2.14-2.10)	50-3.1 (3.15 –3.1)	50-2.1 (2.14-2.10)	50-2.3 (2.31-2.27)
R$_{merge}$[a,b]	0.065 (0.43)	0.070 (0.37)	0.090 (0.37)	0.068 (0.33)
<I>/<σI>[a]	23.1 (2.67)	27.3 (4.9)	34.1 (2.7)	40.2 (3.45)
Completeness (%)[a]	97.6 (96.3)	99.1 (99.4)	99.4 (98.1)	99.9 (99.6)
Redundancy[a]	3.8 (2.3)	6.0 (5.9)	4.8 (3.1)	6.3(4.9)
Beamline	19-ID	19-ID	19-ID	19-ID
Refinement				
Resolution (Å)[a]	50 – 2.1 (2.13-2.09)	50 –3.1(3.18-3.10)	111.7-2.3 (2.36-2.3)	50-2.3 (2.36-2.30)
No. reflections[c]	67072 (3282)	18959 (948)	30554 (1645)	28089 (1404)
R$_{work}$ / Rf$_{ree}$[d]	0.21/0.26	0.24/0.28	0.21/0.26	0.22/0.24
No. atoms				
Protein	6360	6341	3392	3191
Water	164	2	54	108
Ligand	0	0	0	0
Average B-factors (Å^2)	43.0	82.0	59.1	63.4
R.m.s. deviations				
Bond lengths (Å)	0.008	0.004	0.012	0.004
Bond angles (°)	0.935	0.741	1.414	0.780
Ramachandran (%)				
Most favored	99.7	98.5	98.8	98.7
Allowed	0.3	1.5	1.2	1.3

Data Collection	Gp7-1526-1571	Xrcc4-1562-1622	Xrcc4-1590-1657	Xrcc4-1631-1692
Disallowed	0	0	0	0
TLS Groups	16	10	2	4
PDB Accession Number	5CJ1	5CJ4	5CHX	5CJ0

[a] Data in parentheses represent the highest resolution shell.

[b] $R_{merge} = \sum \# I_{hkl} - \bar{I}_{hkl} \# / \sum \# I_{hkl} \#$

[c] Data in parentheses represent the number of reflections used for the calculation of R_{free}.

[d] $R_{factor} = |F_{obs} - F_{calc}| / |F_{obs}|$

Where R_{work} refers to the R_{factor} for the data used in the refinement and R_{free} refers to the R_{factor} for 5% of the data that were excluded from the refinement.

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Table 3

Simulation summary

Model	Trajectory Length (ns)
MyH7-1551-1602[a]	1000
Gp7-MyH7-1551-1602	1000
Xrcc4-MyH7-1551-1603	1400
MyH7-1526-1689	1100
MyH7-1526-1689	520
MyH7-1526-1689	540

[a]Residues 1551-1602 or 1551-1603 were used in the simulation since these constituted the structurally ordered residues in the crystal structure for Xrcc4-1551-1609 (Accession number 4XA4).

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Table 4

Circular Dichroism Theta Values for myosin fusion proteins

Construct	Θ (deg cm^2 dmol^{-1}) at 222 nm
MyH7-1361-1406	−12,733
Gp7-MyH7-1361-1406	−23,247
GCN4-MyH7-1361-1406	−27,766
Xrcc4-MyH7-1361-1406	−14,873