Published in final edited form as:

J Struct Biol. 2009 November ; 168(2): 240–249. doi:10.1016/j.jsb.2009.07.020.

Phosphorylation and the N-terminal Extension of the Regulatory Light Chain Help Orient and Align the Myosin Heads in *Drosophila* Flight Muscle

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Abstract

X-ray diffraction of the indirect flight muscle (IFM) in living *Drosophila* at rest and electron microscopy of intact and glycerinated IFM was used to compare the effects of mutations in the regulatory light chain (RLC) on sarcomeric structure. Truncation of the RLC N-terminal extension (*Dmhc2*^{Δ2-46}) or disruption of the phosphorylation sites by substituting alanines (*Dmhc2*^{S66A, S67A}) decreased the equatorial intensity ratio (I_{20}/I_{10}), indicating decreased myosin mass associated with the thin filaments. Phosphorylation site disruption (*Dmhc2*^{S66A, S67A}), but not N-terminal extension truncation (*Dmhc2*^{Δ2-46}), decreased the 14.5 nm reflection intensity, indicating a spread of the axial distribution of the myosin heads. The arrangement of thick filaments and myosin heads in electron micrographs of the phosphorylation mutant (*Dmhc2*^{S66A, S67A}) appeared normal in the relaxed and rigor states, but when calcium activated, fewer myosin heads formed cross-bridges. In transgenic flies with both alterations to the RLC (*Dmhc2*^{Δ2-46; S66A, S67A}), the effects of the dual mutation were additive. The results suggest that the RLC N-terminal extension serves as a “tether” to help preposition the myosin heads for attachment to actin, while phosphorylation of the RLC promotes head orientations that allow optimal interactions with the thin filament.

Keywords

X-ray diffraction; cross-bridges; myosin regulatory light chain; phosphorylation; extension; work production

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†This article is dedicated to the memory of Mary C. Reedy.

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1. INTRODUCTION

The indirect flight muscles (IFM) of *Drosophila melanogaster* provide an excellent model system for integrated studies of muscle structure and function because of their high degree of structural order and the ease of producing transgenic organisms (Irving, 2006; Maughan and Vigoreaux, 1999; Vigoreaux, 2001). Acto-myosin interactions in IFM and other striated muscles are modulated by the essential light chain (ELC) and the regulatory light chain (RLC) that wrap around the α -helical light chain domain (LCD) of myosin sub-fragment 1 (S1). The RLC is phosphorylated by myosin light chain kinase (MLCK) at conserved serine and threonine residues (Cole et al., 1985; Ikebe and Hartshorne, 1985) with differing effects on acto-myosin interactions depending on the muscle type. Phosphorylation of the RLC in smooth muscle is a primary regulator, leading to large conformational changes and reversible assembly of filaments (Ikebe and Morita, 1991; Trybus and Lowey, 1984). In striated muscles, however, myosin is stably bound with other proteins in well-organized thick filaments, where the influence of the light chains on structure and function is more subtle. Electron microscopy of isolated thick filaments has indicated that phosphorylation of the RLC in striated muscle may increase force development by inducing movement of myosin heads away from the backbone to where they presumably can more readily interact with actin (Levine et al., 1996; Yang et al., 1998). Mechanical measurements indicate that phosphorylation of the RLC increases the sensitivity of force to calcium in skeletal and cardiac muscles and accelerates the rate of force redevelopment (k_{tr}) in skeletal muscle at low levels of activation (Stelzer et al., 2006). The structural mechanisms by which phosphorylation bring about these beneficial effects are not well understood.

The effects of phosphorylation of the *Drosophila* RLC have been investigated by creating mutant flies (*Dmhc2^{S66A, S67A}*) in which alanines were substituted for serines 66 and 67 at conserved myosin light chain kinase-dependent phosphorylation sites (Tohtong et al., 1995). Disruption of the phosphorylation sites produced flies that had impaired flight capability due to reduced power output (Dickinson et al., 1997; Tohtong et al., 1995). No defects in sarcomere structure were seen by electron microscopy after phosphorylation site disruption, so the poor muscle performance was interpreted as the kinetic effects of unphosphorylated RLC that reduced the number of active cross-bridge attachments (Dickinson et al., 1997). Subsequently, x-ray diffraction evidence indicated that in the phosphorylation disruption mutant myosin heads were held closer to the thick filament backbones than in wild type flies (Irving and Maughan, 2000), but detailed information on the disposition of cross-bridges in these muscles was not available.

N-terminal extensions of light chains also influence acto-myosin interaction in a number of muscle types. In mouse hearts, the ELC extensions help determine the contractile properties of the atrium and ventricle. Replacing the ventricular isoform with the shorter atrial isoform through transgenesis significantly increased unloaded shortening velocities determined by *in vitro* motility assays, single fiber mechanics, and whole heart assays (Fewell et al., 1998). The lower velocities associated with the native ventricular isoform is consistent with the notion that the extension acts as an internal load (Lowey et al., 1993a; Lowey et al., 1993b; Sweeney, 1995). That the mammalian ELC extension is able to bind actin (Aydin et al., 2007; Sutoh, 1982; Trayer et al., 1987) or the myosin head (Lowey et al., 2007) under certain conditions is also consistent with the extension creating an internal load. Through its near-neighbor binding, the ELC extension may help maintain a critical inter-filament spacing or head position for optimum force production, a beneficial role *in situ* that could offset any reduced shortening velocity due to drag.

The structural similarities between the N-terminal extensions of the RLC and ELC motivated the design of a *Drosophila* mutant (*Dmhc2^{Δ2-46}*) lacking the N-terminal residues 2–46 of the

RLC extension (Moore et al., 2000). In *Drosophila*, the RLC has a sequence of ~50 residues at the N-terminus (Parker et al., 1985) that is similar to, but longer than, that of the ELC extensions present in mammalian hearts and embryonic and slow skeletal muscles (Arnold et al., 1988). IFM of the RLC truncation mutant appeared normal, but did not generate sufficient oscillatory power to achieve rapid vertical lift, causing the flies to briefly drop towards the ground before flying normally (Moore et al., 2000). It was proposed that the N-terminal extension interacts with actin to increase cross-bridge binding during stretch-activated oscillatory contractions at sub-maximal levels of calcium activation (Irving et al., 2001). Low magnification electron micrographs detected no sarcomere defects in the N-terminal truncation mutant (Moore et al., 2000). However, equatorial x-ray diffraction patterns from the truncation mutant showed an axial spreading of the equatorials away from the center (Irving et al., 2001), suggesting that structural alterations at the level of the myofibrils could be present.

The primary aim of the present study was to examine the interactions between the truncation of the RLC extension and the disruption of the RLC phosphorylation sites using a newly created transgenic fly containing both mutations. An additional aim was to provide a more detailed structural examination of the RLC mutant lines than that reported previously. In this study we examined the IFM of four *Drosophila* lines: the “rescued” control (*Dmhc2*⁺), the phosphorylation site disruption mutant (*Dmhc2*^{S66A, S67A}), the N-terminal truncation mutant (*Dmhc2*^{Δ2-46}) and the newly created dual mutant (*Dmhc2*^{Δ2-46; S66A, S67A}). A combination of small-angle x-ray diffraction of live flies at rest and electron microscopy of muscle fibers was used to compare structural data from live muscle unmodified by isolation or fixation with structural data from isolated intact or glycerinated IFM prepared for thin section electron microscopy. This integrated approach provided a more complete picture of the structural changes at the cross-bridges and sarcomere level than is possible with one technique alone.

2. METHODS

2.1. *Drosophila* strains

Flies were maintained at 22°C in a yeast agar medium. Experiments were performed on the following fly strains: w; P [*mlc2*⁺, w+]; *mlc2*^{E38.13}/*mlc2*^{E38e} (abbreviated as *Dmhc2*⁺), a transgenic line (line 40.1) in which the full length *Dmhc2*⁺ gene is expressed in a null *mlc2*^{E38} background (Warmke et al., 1992); w; P [*mlc2*^{Δ2-46}, w+]; *mlc2*^{E38e}/*mlc2*^{E38.13} (abbreviated as *Dmhc2*^{Δ2-46}), a transgenic line that expresses a truncated version of *Dmhc2*, missing amino acids 2-46 at the NH- terminal (Moore et al., 2000); w; P [*mlc2*^{S66A, S67A}, w+]; *mlc2*^{E38e}/*mlc2*^{E38.13} (abbreviated as *Dmhc2*^{S66A, S67A}), a transgenic line expressing *Dmhc2* with phosphorylation sites serines 66 and 67 replaced by alanines (Tohtong et al., 1995); and w; P [*mlc2*^{Δ2-46; S66A, S67A}, w+]; *mlc2*^{E38e}/*mlc2*^{E38.13} (abbreviated as *Dmhc2*^{Δ2-46; S66A, S67A}), a new dual mutant strain with serines 66, 67 replaced by alanines and lacking amino acids 2-46.

2.2. Generation of *Dmhc2*^{Δ2-46; S66A, S67A} dual mutant transgenic flies

2.2.1. Cloning of dual mutant gene—The PCR template was pCasNDL, the truncated *Dmhc2* construct engineered into a P-element transformation vector by (Moore et al., 2000). The primers used to incorporate the phosphorylation site mutations were:

2341F— 5'CAACTCCTTGTCGTTGGCGAT;

alandrev— 5'CGCGCTGGCGCCGCGGTCTTC;

2880R— 5'AAGATCCTTCAACGCGGATAA;

alandkfw— 5'GAAGACCGCGGCGCCAGCGCG.

Sequence changes coding for alanine are in bold underlined. The PCR mix included 5 units Taq polymerase (Gibco), Taq polymerase buffer, 0.2 mM dNTP's, 20pml/μl of each primer, 1.5 mM Mg Cl₂ and distilled water to complete a 50 μl volume. PCR temperature and time profile included 30 cycles of denaturing at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. After the reaction was complete, the products were digested with BamHI and XhoI. A 132 base pair fragment was ligated to a 10.85 kb gel purified fragment obtained from the XhoI and BamHI digestion of pCasNDL. The new transformation vector, referred to as pCasNDL-ALA, was transformed into *E. coli* and purified using Qiagen plasmid maxi-kit. The alanine substitutions were confirmed by DNA sequencing.

2.2.2. Generation and characterization of transgenic flies—Purified pCasNDL-ALA (400–600 ng) was mixed with 200 ng of helper plasmid pW28.1 in a total volume of 10μl phosphate buffer. This mixture was microinjected into *w¹¹¹⁸* dechorionated eggs following the procedure as described (Moore et al., 2000). Survivors were singly mated to *we* flies and transformants identified by non-white eye color. Chromosome linkage of the transgene was determined by crosses to *ap^{xa}* (*w*; T(2;3)*ap^{xa}*/CyO; TM3 Sb e). A total of five independent insertion lines were mapped to the third chromosome, i.e., the chromosome carrying the endogenous *mlc2* gene. We used the P-element hopping technique (Robertson et al., 1988) to generate additional lines with the transgene in chromosome 2. Virgin (*w*; +; P [*w*+, *Dmlc2^{Δ2-46}*; S66A, S67A], e) females were crossed with *w*; Sp/CyO; Δ2-3 Dr/TM6, Tb males. Male Cy, Dr progeny were then mated to *w¹¹¹⁸* virgin females and the non-Cy, non-Dr males mated to *ap^{xa}* (*w*;T(2;3)*ap^{xa}*/CyO; TM3 Sb e) virgin females. This protocol produced 11 independent lines with a second chromosome transgene insertion.

To generate transgenic lines in a null *Dmlc2* background, we carried out parallel sets of crosses of each second chromosome insertion line to *Dmlc2^{E38.13}*/TM6 Sb e and *Dmlc2^{E38e}*/TM6 Sb e strains. F1 progeny from these crosses were then mated to generate the heterozygote dual mutant (*w*; P [*w*+, *Dmlc2^{Δ2-46}*; S66A, S67A]; *mlc2^{E38.13}*/*mlc2^{E38e}*).

2.3. Preparation of whole live flies for x-ray diffraction experiments

Flies were anesthetized with hydrated CO₂ and a small gauge (~100 mm) tungsten wire was attached between the head and thorax using cyanoacrylate gel glue. The glue held the fly's head and thorax in a stationary vertical plane while the wire was used to hold the fly in the path of the x-rays (Irving and Maughan, 2000). Diffraction patterns from the dorsal longitudinal muscles (DLM) of the live flies were obtained in the resting state (wings folded).

2.4. X-ray diffraction

X-ray patterns were taken on the small-angle instrument at the BioCAT facility (Fischetti et al., 2004) at the Advanced Photon Source. Exposure times were 1 sec at an incident flux of 10¹¹–10¹² photons per second. The distances between pairs of equatorial reflections were converted to *d*₁₀ lattice spacing using Bragg's law, which, for small angles, reduces to *d*₁₀ = 2λL/S, where S = distance from the center of the pattern to a given reflection, L = specimen detector distance (2–3 m), and λ = the wavelength of the x-rays (0.103 nm). Lattice spacing, *d*₁₀, being the perpendicular distance between the lattice planes containing the thick filaments, may be converted to inter-thick filament spacing by multiplying by 2/√3.

Both the equatorial and meridional patterns were converted to one-dimensional projections using the projection tool in the program FIT2D (Hammersley and Riekell, 1989). The intensity data as a function of pixel number was saved as an ASCII format file for later analysis. The peak intensity, widths, and peak separations for the 1,0 and 2,0 equatorial reflections were estimated using a non-linear least squares fitting procedure as described previously (Irving, 1989). In order to obtain good fits, an additional Gaussian peak was included in the model to

account for a broad diffuse reflection arising from the cuticle. This additional peak was not included in a more rudimentary model used in an earlier study of living *Drosophila* DLM (Irving and Maughan, 2000). This omission probably led to less accurate intensity estimates in the earlier study. A change in the ratio of the 2,0 and 1,0 equatorial reflections, I_{20}/I_{10} , was used as a measure of the shift of cross-bridge mass between the thick and thin filaments (Irving, 2006).

The spacings and intensities of the near-meridional 14.5 and 7.2 nm reflections were fit using the multiple peak fitting routines within the program FIT2D (Hammersley and Riekell, 1989). Reflections are referred to as “near-meridional” since there is no intensity on the meridian itself due to the axial 3-fold stagger of the adjacent thick filaments (Squire et al., 2006). The background was estimated as a third order polynomial while the peak positions, widths, and intensities were estimated assuming a Gaussian peak shape. In order to compare intensities from different flies, the intensities from the meridional patterns were normalized to the intensity of the 2,0 equatorial diffraction intensity since the changes in this reflection are small (< 5%) when muscle changes state (Tregear et al., 1998). Changes in the spacing of the 14.5 nm near-meridional reflection were measured as an indicator of the axial displacements of the centers of cross-bridge mass along the thick filament. Changes in the 14.5 nm reflection intensity were used as an indicator of changes in axial cross-bridge orientation. (This intensity will not be sensitive to any azimuthal components of changes in orientation). The intensity will be weakest when the cross-bridges are at oblique angles or distributed over a wide range of axial angles and will increase as the population of cross-bridges becomes more perpendicular to the filament axis (Huxley et al., 1983; Irving and Millman, 1992; Reconditi et al., 2005). Studies of vertebrate muscle indicate that most of the 7.2 nm reflection intensity comes from structures within the backbone (Huxley et al., 2006), but changes in this intensity do not yet have a simple interpretation. The spacing of the 7.2 nm reflection, however, can be used as an indicator of changes in thick filament length (Dickinson et al., 2005; Reconditi et al., 2005).

2.5. Electron microscopy

IFM samples were prepared for transmission electron microscopy according to (Reedy and Beall, 1993) with minor modifications described here. Twenty-four- to forty-eight-hour-old female flies were anesthetized with diethyl ether and mounted in slots formed in modeling clay. The head and abdomen were removed, the cuticle along the dorsal midline was chipped away with tungsten needles, one blade of iridectomy scissors was inserted down the path previously occupied by the gut, the other outside, between the legs, and the ventral thorax was cut in half. The bar of cuticle across the anterior and posterior thorax was cut. The thoraces were gently split down the midline, into hemi-thoraces.

IFM were prepared in two alternate states: in a ‘live’ resting state with membranes intact and in a glycerinated state with membranes solubilized. The glycerinated state allows visualization of myosin heads in electron micrographs (EM’s) and control of the cross-bridge state (relaxed, active or rigor). Glycerinated (“chemically skinned”) IFMs were prepared as described (Reedy et al., 1989). Hemithoraces were incubated 1–2 hrs with 1% Triton X100 in a relaxing solution (pCa 9.0) containing 5 mM MOPS buffer, pH 6.8, containing 150 mM KCl, 5 mM EGTA, 5 mM MgCl₂, and 5 mM Na₂ATP and anti-protease (0.2 mM leupeptin, 0.5 mM PMSF or an anti-protease cocktail (Roche)), followed by an overnight incubation with 50% glycerol in the same buffer at 4°C on a rotator. Next morning, samples were washed with the relaxing buffer with Triton X100 without anti-protease for 1 hr and then 50% glycerol/relaxing solution without Triton for at least 2 hrs. Muscles are stored at either –20°C, in 50% glycerol/relaxing or at –80°C in 75% glycerol/relaxing solution until use.

Hemi-thoraces were put into the desired physiological state by rinsing out the glycerol and completely depleting (rigor) or maintaining (relaxed) MgATP levels. For relaxed preparations,

thoraces were rinsed 4 times in relaxing solution with 5 mM NaAzide substituted for the protease inhibitor described above. For rigor fibers, preparations were rinsed 6 times in rigor buffer (same as relaxing buffer without ATP). Activated fibers were rinsed in activating solution (same as relaxing buffer except at pCa 4.5) for no more than 5 minutes.

For fixations, hemi-thoraces were directly immersed in a freshly prepared fixative consisting of 3% glutaraldehyde and 0.2% tannic acid in the appropriate relaxed, rigor or activating buffer (pH 6.8) for 2 h at room temperature (Fyrberg et al., 1990). After primary fixation, thoraces were rinsed three times for 15 min in buffer and three times for 2 min in 100 mM phosphate buffer (pH 6.0) with 10 mM MgCl₂. Subsequently, the thoraces were immersed for 1 h in ice-cold secondary fixative consisting of 1% osmium tetroxide in 100 mM phosphate buffer and 10 mM MgCl₂ (pH 6.0). After 3 washes in water for 5 min, thoraces were block-stained in aqueous 2% uranyl acetate for 1 h at 4°C and rinsed in H₂O and dehydrated by an ethanol series (50 – 100%). Hemithoraces were infiltrated and some fibers were removed from the hemithoraces before embedding in Araldite 506 mix as described (Reedy et al., 1988). After 3 changes of accelerated Araldite mix, Beem capsules filled with Araldite 506 accelerated mix, were inverted over the hemi-thoraces or individual DLM fibers oriented on polyethylene sheets and polymerized for 48 hrs at 65°C. Ultra thin sections (25–40 nm) were cut with a Diatome diamond knife, picked up on carbon coated grids, stained with 2% aqueous KMnO₄ for 15 minutes, rinsed in Pal's bleach and H₂O, followed by Sato's Lead Stain (Sato, 1968) for 1 minute and rinsed with water. Sections were photographed on S0163 film using a FEI (Philips) 420 electron microscope at 100 kV. Original magnifications ranged from 2,000 to 35,000 X. Negatives were digitized at 800 dpi using a flat bed scanner.

2.6. Statistics

Data are presented as means $\pm$ standard error of the mean. Statistical analysis was performed using SPSS v.14.0 (SPSS, Chicago, IL). Statistical tests were considered significant at the $p < 0.05$ levels. One-way analysis of variance (ANOVA) tests were performed to test for differences between the means of the different strains. If differences were found to be significant, Duncan's post-hoc test was used to determine which means differed. A two-way ANOVA was performed to detect the presence or absence of combined effects or interactions between the RLC N-terminal extension (+/- the RLC N-terminal extension, "Ext effect") and RLC phosphorylation (+/- the phosphorylatable serines, "Phos effect").

3. RESULTS

3.1. X-ray diffraction of live *Drosophila* IFM

Figure 1 compares histograms of the lattice spacings of the thick filaments (d_{10}) (Figure 1A) and the I_{20}/I_{10} equatorial intensity ratios (Figure 1B) from x-ray patterns from the control (*Dmhc2*⁺; n=18), N-terminal truncation (*Dmhc2* ^{Δ 2-46}; n=33), phosphorylation site disruption (*Dmhc2*^{S66A, S67A}; n=36) and the dual mutant (*Dmhc2* ^{Δ 2-46; S66A, S67A}; n=23) flies. Lattice spacings (d_{10}) tended to be lower but were not significantly different in the single mutants (N-terminal truncation or phosphorylation site disruption) when compared to the control line; however, d_{10} was significantly reduced (~0.9 nm) in the dual mutant (Figure 1A) leading to the significant two-way ANOVA extension (Ext) and phosphorylation sites (Phos) effects as well as the significant interaction between the extension and phosphorylation sites (Ext by Phos). Equatorial intensity ratios (I_{20}/I_{10}) for the single mutations were significantly reduced (14–16%) compared to control with a larger reduction (31%) in the dual mutation (Figure 1B), indicating one or both myosin heads were further from the thin filaments in the mutant lines under resting conditions, in agreement with other studies (Lowey et al., 1993b; Taylor et al., 1999; Trybus and Lowey, 1984). Analysis of the equatorial intensity ratios by two-way ANOVA indicated that the effects of removing either the extension (Ext) or phosphorylation

sites (Phos) were statistically significant, but no interactions between the truncation and the phosphorylation disruption were detected, i.e., these mutations appear to exert their effects independently. However, our results indicate that the mutations have an additive effect on the equatorial intensity ratios since the dual mutant has a significantly larger reduction than the single mutants.

The axial spacings of the ~14.5 nm near-meridional reflections of the control (n=8), N-terminal truncation (n=24) and dual mutants (n=7) showed no significant differences, indicating that the axial positions of the center of mass of one or both of the myosin heads are similar along the thick filament under resting conditions (Figure 2A). In contrast, the axial spacing of the 14.5 nm reflection in the phosphorylation site disruption mutant (n=5) was significantly reduced compared to the other lines (Figure 2A). The interaction (Ext by Phos) effect reported by two-way ANOVA indicates an interaction between the N-terminal extension and the phosphorylation sites on the RLC in their effects on the 14.5 spacing. The 14.5 nm reflection intensities ($I_{14.5}$) of the truncation mutant (Figure 2B) were not significantly different from the control, but those of the phosphorylation site disruption and dual mutants were significantly reduced (by ~50–60%) compared to the control. Analysis by two-way ANOVA indicated that the decrease in 14.5 nm reflection intensity when removing the phosphorylation sites was significant. This suggests that the myosin head orientations in phosphorylation disruption mutants (*Dmhc2^{S66A, S67A}* and *Dmhc2^{Δ2-46; S66A, S67A}*) had a greater amount of axial disorder compared to IFM with normal phosphorylation sites (*Dmhc2⁺* and *Dmhc2^{Δ2-46}*).

There were no significant differences in the spacings of the 7.2 nm near-meridional reflection in all four fly strains (control, n=8; N-terminal truncation, n=24; phosphorylation site disruption, n=5; dual mutant, n=11) under resting conditions (Figure 2C), indicating that the thick filament backbone periodicity is the same in all four strains (Dickinson et al., 2005; Reconditi et al., 2005). However, two-way ANOVA showed that the increases in the 7.2 nm intensity (Figure 2D) upon deleting the extension (*Dmhc2^{Δ2-46}* and *Dmhc2^{Δ2-46; S66A, S67A}*) were significant (Ext effect), suggesting that removal of the extension may lead to an increase in the ordering of the packing of myosin molecules in the backbone in a way that strengthens the axial periodicities giving rise to the 7.2 nm reflection.

3.2. Electron Microscopy

In order to examine cross-bridge structure, we viewed single filament layers of alternating myosin and actin filaments (myac layers) connected by cross-bridges. IFMs were prepared “live” or glycerinated in order to improve visualization of myosin heads in EM’s and to control cross-bridge state (relaxed, active or rigor). Notably, the x-ray diffraction results indicated no differences in the 14.5 nm reflection spacing or intensity between the N-terminal deletion mutant and control, suggesting no changes in myosin head axial orientation, so this mutant was not examined in longitudinal sections under relaxed, active or rigor conditions.

Figures 3A and 3B compare EMs of longitudinal sections from the control and the phosphorylation disruption mutant under relaxed conditions, where significant changes in myosin head axial disposition were noted by x-ray diffraction. In both cases, cross-bridges adopt a large range of angles relative to the thick filament that reflects the axial stagger of the cross-bridge origins on adjacent thick filaments due to their 3-fold superlattice arrangement (Squire et al., 2006). These high-resolution results confirm previous findings from lower resolution EMs (Dickinson et al., 1997) that the ultrastructure is similar between the control and phosphorylation disruption mutant under relaxed conditions.

Under calcium activated conditions, fibers from the control line (Figure 3C) showed cross-bridge pairs at most 38.7 nm repeats while the phosphorylation disruption mutant (Figure 3D) often showed only a single cross-bridge per 38.7 nm repeat (on alternating sides of the thin

filament as one follows along the thick filament) or no cross-bridge pairs on some repeats. These high-resolution EMs qualitatively show fewer active cross-bridges are binding in the phosphorylation mutant during isometric contraction whereas previous findings based on lower resolution EMs suggested no differences under calcium activated conditions between these two lines (Dickinson et al., 1997).

Cross-bridges in fibers from the control line in the absence of nucleotide (rigor) form regularly angled cross-bridge pairs at every 38.7 nm repeat along the thin filament (Figure 3E). High resolution measurements were not repeated since previous lower resolution images clearly show that rigor cross-bridges in IFM from the phosphorylation disruption mutant appear equally as well ordered as those in the control line in longitudinal sections (Dickinson et al., 1997). A number of the rigor cross-bridges in IFM from the dual mutant, however, deviate from the uniform cross-bridge angles of rigor “chevrons”, indicating structural defects at the level of the interactions of individual cross-bridges (Figure 3F).

A less common view of IFM that reveals even small irregularities in sarcomere and cross-bridge structure is provided by ultra-thin (15 nm) cross-sections of rigor IFM that include only single (14.5-12.9 nm) levels of cross-bridges and their origins. In rigor, the helical progression of well-formed “flared X” cross-bridges is a stringent test of the regularity of the filament and cross-bridge lattice. In the N-terminal truncation mutant, the expected ~60° rotation of well-formed “flared X” levels is observed across the entire myofibril out to the uniform periphery (Figure 4). These micrographs are indistinguishable from those from wild-type IFM (not shown). Thus, neither the absence of the RLC extension (as examined in cross-sections; Figure 4) nor the disruption of the phosphorylation sites (as examined in longitudinal sections; Dickinson et al., 1997) appear to prevent the formation of well-ordered rigor cross-bridges whereas the presence of both mutations in the dual mutant does lead to disruption of rigor cross-bridges (Figure 3F).

In contrast to control, the N-terminal truncation mutant (Dickinson et al., 1997), and the phosphorylation site mutant (Moore et al., 2000), many of the myofibrils from the dual mutant showed fraying and mis-register of filaments and Z-bands at the periphery of the sarcomeres, shown in longitudinal view under “live” conditions in Figure 5. Some sarcomere and filament length variation is also evident.

4. DISCUSSION

4.1. Role of the N-terminal extension of the RLC on insect IFM

The interaction of myosin heads with actin in IFM appear to occur at well-defined “target zones” on the thin filament (Tregear et al., 1998). We previously proposed that the RLC extension enhances oscillatory work output by serving as a structural linkage that helps pre-position myosin heads near these actin targets (Irving et al., 2001). This hypothesis was based on the decreased flight performance and kinematics in the flies with the N-terminal extension truncation (*Dmhc2^{Δ2-46}*) compared to control (Moore et al., 2000), in conjunction with the finding of a decreased intensity ratio (I_{20}/I_{10}) in the x-ray diffraction patterns from flies with the N-terminal extension truncation (Irving et al., 2001). This lower intensity ratio in the truncation mutant implies that, when the extension is absent, myosin heads are, on average, less associated with the thin filament (Irving, 2006).

In the present study we again found that the I_{20}/I_{10} decreases in the N-terminal extension truncation compared to control. We also found similar intensities of the 14.5 nm reflection in the control and truncation mutants, which suggests that the heads have a similar range of axial orientations. Based upon these data, we speculate that since there is no RLC extension to tether the heads to the thin filament, the heads are further away from their target zones and therefore

take longer to find their targets on the thin filament (Figure 6A and 6B). We further speculate that the decrease in ability to find their targets on the thin filament will alter myosin kinetics in the truncation mutant, driving the decreases in whole fly flight characteristics. Notably, electron microscopy results indicate that the myosin heads from the truncation mutant are still able to strongly bind to the thin filament, if given enough time, since their rigor heads are indistinguishable from control.

The exact nature of the putative structural linkages is not known. One possibility is suggested by analysis of the amino acid sequences of the regulatory light chain and two thin filament-associated proteins: TmH (the longer of two isoforms of tropomyosin in *Drosophila* IFM) (Mateos et al., 2006) and TnT (a unique subunit of the troponin complex in IFM) (Fyrberg et al., 1990). Positive charges near the N-terminal end of the RLC extension (Lysines) (Nongthomba et al., 2007; Parker et al., 1985) could interact electrostatically with negative charges (Glutamates) on the extended C-terminus of TmH or TnT to form transient linkages tethering the heads to the thin filament (Nongthomba et al., 2007).

4.2. Role of phosphorylation of the RLC in insect IFM

Previous electron microscopy of isolated thick filaments of skeletal muscle suggests that, upon phosphorylation, heads appear disordered on the surface of the thick filament and extend to higher radii (Levine et al., 1995; Levine et al., 1996). Dephosphorylated heads, on the other hand, are more closely associated with the thick filament backbone and tilted with respect to the filament axis (Levine et al., 2001), which presumably results in a less favorable presentation of heads to actin. High resolution reconstructions obtained by single particle analysis of electron micrographs of isolated thick filaments from Tarantula muscle (a RLC phosphorylation regulated muscle, like smooth vertebrate muscle) (Woodhead et al., 2005) indicate that in the resting state, one head of myosin interacts with a corresponding head in an adjacent pair, which presumably stabilizes that head in an “off” state (Burgess et al., 2007; Jung and Craig, 2008; Wendt et al., 2001). It has been suggested that such interactions may be a general feature of myosin II based motile systems (Jung and Craig, 2008; Zoghbi et al., 2008).

In contrast, the *Lethocerus* IFM has a resting myosin head configuration that appears to be very different from other muscles. Using a structural model based upon x-ray diffraction data (Squire et al., 2006), one head of a pair of a resting myosin molecule in *Lethocerus* is roughly perpendicular to the thick filament long axis while the other curves around the thick filament surface to nose against the proximal neck of the projecting head of the neighboring myosin molecule. i.e. the interactions that stabilize the relaxed structure are inter-molecular rather than intra-molecular (see also Oshima et al 2007) (Oshima et al., 2007) who also proposed intermolecular contacts between heads in living, resting frog muscle). There are currently no EM reconstructions from isolated thick filaments of any IFM, so it is not clear how much of the differences in resting myosin head configuration are due to variations between species or to the perturbing effects of the presence of thin filaments in intact myofibrils as opposed to isolated thick filaments. It is also not clear how applicable the current model for the *Lethocerus* thick filaments (Squire et al., 2006) are to *Drosophila* thick filaments, where the myosin head distributions are clearly very different (compare Figure 3A and 3B with Figure 7a of Reedy et al., 1992) as is the arrangement of the thick filaments themselves. In *Lethocerus* muscle, the thick filaments are in a simple lattice where the thick filaments all have exact translational registry whereas in *Drosophila* model there appears to be a super-lattice where there is a three fold axial stagger between adjacent thick filaments (Squire et al., 2006). In summary, there are a number of potential structural mechanisms for inhibiting the mobility of unphosphorylated myosin heads that involve interactions of the heads with either

the backbone, each other or within themselves. Determination of which mechanism predominates in *Drosophila* IFM must await further studies.

Our x-ray diffraction results are consistent with the view that phosphorylation acts as a modulator of contraction in *Drosophila* IFM, as with other non-regulated myosins, and not as a simple “on-off” switch (Sweeney et al., 1993) in that the structural changes between control and the phosphorylation disruption mutants are subtle. The decreased intensity of the 14.5 nm reflection indicates that one or both of the heads in the phosphorylation site disruption mutant (*Dmhc2^{S66A, S67A}*) adopt more oblique angles, or a broader axial distribution of angles, than in either the control or the N-terminal truncation mutant (Figure 6C). The RLC extensions in the IFM from the phosphorylation site disruption mutants would still be able to link to the thin filament, but the dephosphorylated heads would adopt a wider range of angles relative to the thick filament backbone, as indicated by the reduced intensity of the 14.5 nm reflection. As a consequence, the center of mass of myosin heads would be, on average, further away from the thin filament as indicated by the reduced equatorial ratio (I_{20}/I_{10}).

The slightly (~0.7%), but significantly, smaller spacing of the 14.5 nm reflection indicates that the axial separation of the crowns of myosin heads is shorter in the phosphorylation disruption mutant suggesting a slight change in the myosin packing structure. The mean value of the 7.2 nm reflection spacing is also shorter (Figure 2C), albeit not significantly (because of the large error bar), consistent with this notion. We speculate that this change in filament structure could be due to interactions of the light chains with the backbone as suggested by EM results of skeletal muscle with unphosphorylated light chains (Levine et al., 2001). It could also be due to heads adopting an “off” configuration due to intramolecular interactions within myosin (Craig and Woodhead, 2006) or to intermolecular interactions (AL-Khayat et al., 2003) as discussed above. Alternatively, the change in myosin packing could be due to a small effect of the mutation on the assembly of the thick filaments during development.

Close inspection of electron micrographs in the present study indicate that fewer myosin heads in the IFM from the phosphorylation site disruption mutant are in contact with actin under steady-state contracting conditions. This suggests that the ability of the myosin head to form productive acto-myosin interactions is impaired. The electron micrographs of the IFM in rigor indicate that the myosin heads, given enough time, will form rigor cross-bridges in the phosphorylation site mutant (Dickinson et al., 1997). Because of the wider range of starting angles of the myosin heads, along with possible “docking” of heads in the “off” state, both of which may be expected to alter head mobility, we speculate that it takes longer for the heads to find their target zones on the thin filaments in the phosphorylation site mutants compared to controls or the N-terminal extension mutants. Thus, it is not surprising that phosphorylation site disruption mutants have impaired flight capability and that fibers isolated from these flies show reduced power output (Dickinson et al., 1997; Tohtong et al., 1995).

4.3. Effect of the dual mutation on the structure of *Drosophila* IFM

The dual mutation line (*Dmhc2^{Δ2-46; S66A, S67A}*) was created to determine whether truncation of the RLC N-terminal extension and the disruption of the RLC phosphorylation sites have additive or compensatory effects on myosin structural properties and function. Previous studies using the single mutations indicate that the truncation of the N-terminal extension (Moore et al., 2000) and disruption of the phosphorylation sites (Dickinson et al., 1997) have distinct phenotypes.

Our data indicates that the phenotype of the dual mutant is largely a summation of the two individual mutations. The N-terminal extension truncation alone reduces the intensity ratio (I_{20}/I_{10}) by 14%, with no change in $I_{14.5}$ with respect to control. Phosphorylation site disruption reduces the intensity ratio by 16% and $I_{14.5}$ by ~60% with respect to control. The two mutations

together reduce the intensity ratio by 34% and $I_{14.5}$ by ~50%. Thus, lacking both the tethering ability of the extension to keep the heads near the thin filament and the phosphorylation of the RLC serines to orient the heads with respect to the thin filament, the heads maintain positions away from the thin filament (Figure 6D). This would be consistent with the reduced ability to form normal well-ordered rigor cross-bridges that we observed in electron micrographs. Together, these results suggest that the RLC N-terminal extension and phosphorylation sites work synergistically in regulating myosin activity in IFM.

The 14.5 nm spacing in the dual mutant does not appear to be an average of the two individual mutations. While the phosphorylation site disruption mutation has a statistically significant 0.6% decrease in 14.5 nm spacing, this spacing in the dual mutant is unchanged from controls. The slightly altered packing structure adopted by myosin heads in the phosphorylation site disruption mutants is presumed to be stabilized by interactions between the RLC and the thick filament backbone and/or neighboring heads as discussed above. Removing the N-terminal extensions of the RLC's appears to disrupt these interactions, allowing the heads to adopt configurations with their centers of mass closer to the normal values.

4.4 Findings of current study versus previous studies

We found two major differences in the findings between our current and previous studies. First, while the lower resolution EMs of previous studies indicated that the active cross-bridge arrays of the phosphorylation site disruption line were similar to those of the control (Dickinson et al., 1997), the higher resolution EMs in this study revealed fewer cross-bridges were formed in Ca^{2+} -activated myofibrils in the phosphorylation site mutant. Second, while previous studies found a slightly reduced inter-filament lattice spacing (d_{10}) in both the N-terminal extension truncation (Irving et al., 2001) and phosphorylation site mutants (Irving and Maughan, 2000) compared to control, the present study did not. However, the control line used in the previous two studies was different from our current study because the line used previously was no longer available; therefore, the simplest and possibly only reasonable explanation for the discrepancy is that two control lines had different physical properties. It is important to recognize, however, that our primary findings in this study are based on measures other than inter-filament lattice spacing, and that the structural details obtained by other measures were similar in both studies.

4.5. Summary

The goal of this research was to gain structural insight into the role of distinctive features of the regulatory light chain (RLC) on overall myofibrillar structure and function in *Drosophila* IFM. Our results suggest that 1) phosphorylation of the serines at positions 66 and/or 67 help orient the heads with respect to the thin filaments by restricting the range of angles the head can make relative to the thick filament backbone, and 2) the N-terminal extension helps align the heads with respect to the target zones on the thin filament. Together, the presence of the N-terminal extension and phosphorylation of the conserved serine residues optimize flight performance in *Drosophila*. Mechanical data consistent with these interpretations will be presented elsewhere (Miller et al., in preparation).

Acknowledgments

We are grateful to Dr. David Gore and the BioCAT staff for help with beamline setup at the Advanced Photon Source (APS). This work was supported, in part, by National Institutes of Health (NIH) grant R01 HL68034 (to DWM). Use of the APS was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Energy Research, under Contract No. W-31-109-ENG-38. The Biophysics Collaborative Access Team (BioCAT) is a U.S. NIH-supported Research Center (RR08630 to TCI). The content is solely the responsibility of the authors and does not necessarily reflect the official views of the NIH.

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Figure 1.

d_{10} lattice spacings (A) and intensity ratios (I_{20}/I_{10}) (B) compared in four strains of living transgenic flies under resting conditions. *, † and § indicate mean values significantly different ($p < 0.05$) from the control line ($Dmhc2^{+}$), N-terminal truncation mutant ($Dmhc2^{\Delta 2-46}$) and phosphorylation site disruption mutant ($Dmhc2^{S66A, S67A}$), respectively. Ext, Phos and Ext by Phos indicate a significant difference ($p < 0.05$) by two-way ANOVA between groups with normal N-terminal extension vs. those with N-terminal truncation, between groups with normal phosphorylation sites vs. those with phosphorylation site disruption and a significant interaction between extension and phosphorylation sites, respectively. Error bars represent SEM.

Figure 2.

Comparison of the backbone and myosin head structure of the four strains of transgenic flies. Panels A and C show the spacing of the 14.5 and 7.2 nm reflections. Panels B and D show the relative intensities of the 14.5 and 7.2 nm reflections normalized to the 2,0 equatorial intensity. *, † and ¶ indicate mean values significantly different ($p < 0.05$) from the control line ($Dmlc2^{+}$), N-terminal truncation mutant ($Dmlc2^{\Delta 2-46}$) and the dual mutant ($Dmlc2^{\Delta 2-46; S66A, S67A}$). Ext, Phos and Ext by Phos indicate a significant difference ($p < 0.05$) by two-way ANOVA between groups with normal N-terminal extension vs. those with N-terminal truncation, between groups with normal phosphorylation sites vs. those with phosphorylation site disruption and a significant interaction between extension and phosphorylation sites, respectively. Error bars represent SEM.

Figure 3.

Cross bridge structure in *Drosophila* IFM. Longitudinal 25 nm myac layer sections of glycerinated IFM in relaxing, activating and rigor states. (A) Relaxed control (*Dmhc2*⁺) and (B) relaxed phosphorylation site disruption mutant (*Dmhc2*^{S66A, S67A}) appear very similar, with both showing the normal wide distribution of angles (arrows). (C) Control and (D) phosphorylation site disruption mutant in isometric calcium activation buffer. The control shows a pair of cross-bridges every 38.7 nm repeat (arrows), while the phosphorylation site disruption mutant frequently shows only one cross-bridge per 38.7 nm repeat (arrows). (E) Control IFM in rigor shows regularly angled "chevrons" (arrows) every 38.7 nm repeat. (F) The dual mutant (*Dmhc2*^{Δ2-46; S66A, S67A}) in rigor shows some bridges with more variable

bridge angle (arrows) than normal rigor. Z-band towards the bottom in all pictures. Bar = 39 nm.

Figure 4.

Ultra-thin cross sections (15 nm) of the truncation mutant (*Dmlc2*^{Δ2-46}) in rigor demonstrate the excellent regularity of the cross-bridge and filament lattice. A) View of an entire myofibril in slightly oblique section showing the regular rotation of cross-bridge levels forming “stripes” and the “smooth” periphery (Bar = ~250nm). B) Single rigor cross-bridge levels cropped and rotated “upright” show cross-bridges binding to four of the six actins surrounding each thick filament, forming the flared X, flanked by actin at the level of troponin. View is toward Z-line. Inset: diagram of a flared X. Thick filaments are larger empty circles, flanking thin filaments are black, sigmoids are lead cross-bridges, zig-zags are rear cross-bridges. Bar = ~50 nm. C) A small area where the flared X’s are all oriented identically.

Figure 5.

Electron micrographs of ~30 nm longitudinal sections of the dual mutant (*Dmlc2*^{Δ2-46}; S66A, S67A). (A) Rigor myofibrils showed a well-ordered core, but small bundles of loose, misaligned filaments, Z- and M-bands (arrows) marred the regularity of the sarcomeres. (B) "Live" IFM myofibrils in contact with numerous mitochondria also showed misregistered filaments and Z-bands (arrows). Bars = ~1 micron.

Figure 6.

Schematic illustration of the proposed interactions of the RLC with the thin filament. A) When the phosphorylation sites (red filled circles) and extension are present (*Dmlc2*⁺) the myosin head is held in close proximity to the thin filament and in a relatively restricted angular range (axially), favorable for acto-myosin interaction. B) When the extension is removed (*Dmlc2*^{Δ2-46}) and the phosphorylation sites are present the head maintains its angular orientation but are further from the thin filament. C) When phosphorylation is prevented (*Dmlc2*^{S66A, S67A}), as shown by filled black circles, the head orientation changes even though the extension, acting as a tether, keeps the head in close proximity to the thin filament. D) When both phosphorylation and the extension are removed in the dual mutant (*Dmlc2*^{Δ2-46; S66A, S67A}) the range of myosin head angles increases and proximity to the thin filament decreases, so that acto-myosin interaction is inhibited. Note that the 14.5 nm reflection intensity will be most sensitive to the axial component of any changes in head orientation, Thus the changes in orientation shown in panels C and D, phosphorylation sites in are absent indicate only the axial component of such changes although azimuthal components cannot be excluded.