

NATURE'S STRATEGY FOR OPTIMIZING POWER GENERATION IN INSECT FLIGHT MUSCLE

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1. INTRODUCTION

The most important mechanical function of the actomyosin motor in striated muscles is to generate power, particularly in systems that operate in an oscillatory manner. Since all striated muscles have the same basic motor protein (myosin II), the question arises as to what structural modifications of the sarcomere have evolved to allow oscillatory work and power generated by the motor proteins to not only be amplified, but also to be so well tuned to the operating frequency of the muscle system.

In this chapter we discuss key structural features of the myofilament lattice that have evolved to enhance oscillatory work and power output, using *Drosophila* indirect flight muscle (IFM) as an example. Insect flight muscle is particularly endowed with power-enhancing features since insect flight requires higher power output per gram body weight than other forms of animal locomotion (Marden, 2000; Tregear, 1983). These features include 1) strengthening the weak links of the sarcomere, 2) orienting the head of the myosin molecule for optimum power generation, and 3) modifying the kinetics of the myosin head. We focus on alternative forms of myosin II and proteins associated with the thick filament that serve these functions (**Fig. 1**), particularly those in which mutations have been generated and whose muscle properties have been examined *in vivo* and *in vitro*.

2. PROTEINS AND UNIQUE SEQUENCES THAT MODULATE POWER OUTPUT

Table 1 summarizes thick filament proteins of *Drosophila* IFM in which mutations have been generated and studied by sinusoidal length perturbation analysis (Dickinson et al., 1997; Maughan et al., 1998). The effect of each mutation on the resonance frequency of the flight system (i.e., the wing beat frequency) and muscle power are indicated, as are the primary mechanisms most likely responsible for modifying wing beat frequency (WBF) and muscle power. While WBF and frequency of maximum muscle power output are correlated in native systems (Molloy et al., 1987), this is not always the case in situations where one part of the system has been re-engineered genetically. For example, one mutant may show significant reductions in both WBF and muscle power (RLC *Mlc2*^{S66A,S67A}), while another may show

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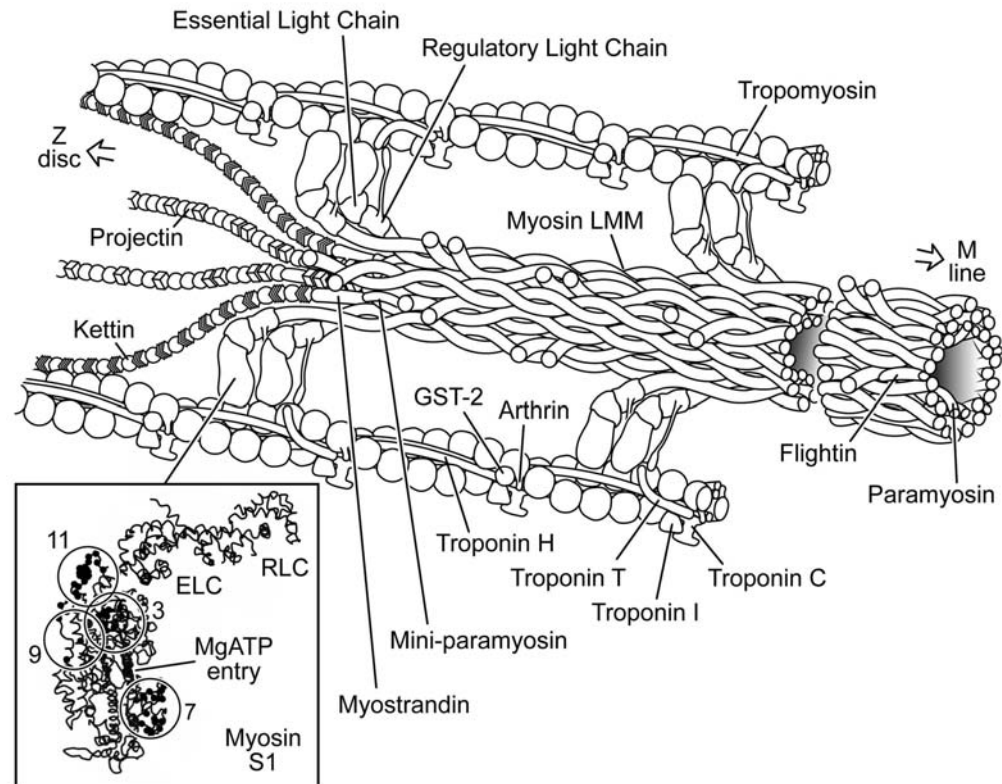


Figure 1. Schematic illustration of a portion of the *Drosophila* myofilament lattice at the A-I junction. Aspects of the lattice (including positions of myostrandin, arthrin, GST-2, and flightin) are highly speculative. Flightin is shown located in a segment of the thick filament further from the end, as determined by immuno-electronmicroscopy (Reedy et al., 2000). Call out: the closely related 3D structure of chicken myosin (Bernstein and Milligan, 1997), where the circles denote the alternatively spliced regions encoded by exons 3, 7, 9 and 11 of the *Drosophila Mhc* gene.

elevated WBF with reduced muscle power (paramyosin pm^{S18A}). The different outcomes reflect different mechanisms that come into play, depending on the protein and site of the mutation and the fly's strategy, voluntary or otherwise, in responding to the challenges of the mutation.

Myosin. Consider first the *myosin heavy chain* mutants, specifically those located in subunit 1 (S1), the motor head. What structural regions of the head determine power output? This question can be addressed in *Drosophila* by taking advantage of the fact that there is only one *Mhc* gene (compared to up to 9 separate MHC genes in mammals). The one gene consists of 19 exons, 5 of which are alternatively spliced (exon 3, 7, 9, 11 and 15: George et al., 1989) and one, exon 18, that is either included or excluded. Four of these exons encode regions of the S1 head which, is the the region of the molecule most likely to determine

kinetics (Rayment et al., 1993). Thus the question of optimizing power output can be initially reduced to addressing which regions, or combination of regions, contribute to the differences in myosin isoform functional properties.

Drosophila melanogaster is an exceptionally useful experimental preparation for myosin structure/function studies. The availability of MHC null mutants and powerful molecular genetic tools allow for the transgenic replacement of specific amino acids or regions of the molecule in the flight and jump muscles, often with dramatic results (Bernstein et al., 1993). Whole isoform substitutions produce particularly striking outcomes (Swank et al., 2002a,b; Littlefield et al., 2003). Substituting the embryonic isoform (EMB) for the native indirect flight muscle isoform (IFI) transforms the IFM from a high power-generating muscle that works optimally at high oscillation frequencies to a lower power-generating muscle that works optimally at low oscillation frequencies.

Single region substitutions also produce large changes in fiber kinetics, although the differences are less striking than observed for whole isoform substitutions. The region encoded by exon 11 (Fig. 1, inset) includes the “converter”, an important structural link between the catalytic part of the molecule and the light chain-draped lever arm that conveys the torque to the myosin rod. Skinned fiber experiments show that exchanging alternative versions of this region affects power production (P) and the frequency of maximum power production (f). Expressing the EMB-IC myosin chimera (made by exchanging the IFI converter into EMB myosin) in IFM fibers increased P and f 2-fold compared to IFM fibers expressing EMB (i.e., *EMB-IC* vs. EMB; Table 1). Conversely, fibers expressing the chimera made by the opposite exchange (replacing the native converter with the EMB converter) reduced P by a third and f by one half compared to native IFM (i.e., *IFI-EC* vs. *IFI*; Table 1). Preliminary experiments suggest other variable regions do not play as great a role in determining myofibril kinetics (Miller et al., 2003; Swank et al., 2002a; Swank et al., 2004), supporting the hypothesis that the converter region is a strategic location.

Light chains. The myosin *essential* and *regulatory light chains* hug the α -helical backbone of S1, reinforcing and stiffening the lever that extends from the catalytic head piece (Fig. 1). A stiffer lever enhances transmission of torque and power to the myofilaments. While little is known of the essential light chain's role in *Drosophila* outside this function, much is known about the modulatory features of the regulatory light chain (RLC). *Drosophila* RLC has two conserved myosin light chain kinase-dependent phosphorylation sites. Disruption of these sites (*Dmlc2*^{S66A,S67A}; Table 1) markedly alters flight ability by reducing oscillatory power output of the IFM (Tohtong et al., 1995). Sinusoidal length perturbation analysis (Dickinson et al., 1997) combined with *in vivo* X-ray diffraction studies (Irving and Maughan, 2000) established that the reduced power output is due to reduced recruitment of cross-bridges into the work-producing pool rather than to changes in the kinetics of actively cycling cross-bridges. The key observations are the relatively high I_{20}/I_{10} intensity ratio observed in X-ray diffraction patterns from live flies at rest (indicating the close proximity of the myosin heads to the thin filament), the relatively small increase in I_{20}/I_{10} intensity ratio observed during the wing beat (indicating little additional lateral movement of the heads toward the thin filament), and the significant reduction in I_{20}/I_{10} intensity ratio in the phosphorylation site mutant (indicating a shift in myosin heads toward the thick filament

backbone). It is tempting to speculate that the phosphorylated RLC maintains the cross-bridges in an extended position (roughly perpendicular to the thick filament axis (AL-Khayat et al., 2003), thereby helping to maintain a critical inter-filament spacing or head position for optimum force and oscillatory work production, as proposed for phosphorylated RLC in vertebrates (Sweeney et al., 1993).

IFM oscillatory power output in *Drosophila* is also enhanced by an N-terminal extension of the RLC that has a counterpart in vertebrate ELC (Bhandari et al., 1986; Fewell et al., 1998; Sweeney, 1995). In flies lacking nearly all 48 amino acids of the N-terminal extension (*Dmhc2^{Δ2-46}*; Table 1), skinned IFM near *in vivo* lattice spacing exhibit significantly reduced net oscillatory work output at submaximal calcium activation (Irving et al., 2001), a deficit that could account for an observed flight impairment (Moore et al., 2000). Preliminary nuclear resonance experiments using bacterially-expressed *Drosophila* RLC (referred to in Irving et al., 2001) provide evidence for a N-terminus RLC-actin interaction similar to that reported earlier for ELC (Prince et al., 1981; Trayer et al., 1987), shown by others to be between specific basic residues near the ELC N-terminus and C-terminal residues of the actin monomer (Andreev et al., 1999; Sutoh, 1982). Again, it is likely that, like the phosphorylation of RLC residues 66 and 67, the N-terminal extension helps maintain a critical inter-filament spacing and/or pre-positions the head for optimum force production and oscillatory work output, as proposed for the ELC extension in vertebrate cardiac muscles (Irving et al., 2001; Sweeney, 1995).

Light meromyosin and other myosin-associated proteins. Apart from myosin S1 and the light chains, the rod portion of myosin and other proteins associated with the myosin rod play major roles in determining power output, especially the efficiency with which power is transmitted to the ends of the sarcomere. Because the myofilaments are reinforced by special proteins (or special adaptations of conserved proteins), the filaments are very stiff and optimized for efficient force transmission, yet compliant enough to offer effective elastic energy storage. The insect IFM thick filament is thicker than its vertebrate counterpart, with 4-fold rotational symmetry and 4 double-headed myosins per crown (of which 2 are shown in the plane of Fig. 1) compared with 3 in vertebrates. The primary structural element of the thick filament is **light meromyosin** (LMM), i.e., the long coiled-coil segment of the myosin molecule, with periodic charges that electrostatically bind one LMM alongside another. The greater stiffness of insect thick filaments follows simply from the fact, stated in engineering terms, two spring-like viscoelastic elements side-by-side are stiffer than one, especially if they are cross-linked.

Additional stiffening of the thick filament results from annealing adjacent LMMs along their length, just as a set of parallel short springs in series is stiffer than a set of parallel long springs. **Flightin** (Fig. 1) may be responsible for annealing, or spot-welding LMMs to one another, by bridging myosin pairs by ionic bonds to form subfilaments, and/or by electrostatic interactions with the S2 hinge to reinforce this particularly flexible part of the myosin rod (Reedy et al., 2000; Vigoreaux et al., 1998). Binding of flightin to LMM and the susceptibility of the S2 hinge to proteolysis in IFM with mutations that prevent flightin accumulation support this view (Kronert et al., 1995; Reedy et al., 2000; Ayer and Vigoreaux, 2003). A flightin null (*fln⁰*; Table 1) and myosin rod mutant (*Mhc^{I3}*; Table 1) with

Table 1. Thick filament proteins of *Drosophila* IFM in which mutations have been studied by sinusoidal analysis.

Protein	Mutant	Mutation	Δ WBF @22°C (mutant/control)	Δ muscle power (<i>P</i>)*	Δ muscle kinetics (<i>f</i>)	Δ muscle stiffness (<i>Y</i>)*	Reference(s)
Projectin	<i>bt^D/+</i>	C-terminal truncation	1.10	n.s.d.	1.44	0.63	Vigoreaux et al., 2000
MHC S1	<i>EMB</i> <i>IFI-EC</i> <i>EMB-IC</i>	EMB myosin substitution EMB converter in IFI IFI converter in EMB	none n.s.d. none	0.23 0.68 0.52	0.13 0.49 0.29	1.53** 1.67** 1.76**	Swank et al., 2002b; Littlefield et al., 2003
MHC rod	<i>Mhc⁶</i> <i>Mhc¹³</i>	R1559H E1554K	N.A. N.A.	0.12 0.03	0.61 0.38	0.76 0.18	Henkin et al., 2004 Henkin et al., 2004
Paramyosin	<i>pm^{S18A}</i> <i>pm^{S-A4}</i>	S18A S9A,S10A,S13A,S18A	1.07 n.s.d.	0.58 0.72	n.s.d. n.s.d.	0.69 0.75	Hao et al., 2004 Liu et al. 2002
RLC	<i>Mlc2^{S66A,S67A}</i> <i>Mlc2^{Δ2-46}</i>	S66A,S67A Δ 2-46	0.83 n.s.d.	0.09 0.55	n.s.d. n.s.d.	0.12** 0.60	Tohtong et al., 1995 Moore et al., 2000
Flightin	<i>Df(3L)fn¹/+</i> <i>fn^o/fn^o</i>	deficiency null	n.s.d. none	n.s.d. virt. none	1.78 0.64	0.39	Vigoreaux et al., 1998 Henkin et al., 2004

n.s.d. = no significant difference

Power (*P*) and dynamic stiffness (*Y*) measured at the frequency of maximum work output (*f*).

Fractions refer to value of index of mutant divided by that of wild type control at pCa 5.

N.A. = flies ~ ½ hr old to avoid time-dependent degradation of the IFM; not capable of flight so soon after eclosion.

* adjusted for any change in number of myofilaments per cross-sectional area of skinned fiber.

** ratio of viscous moduli measured at frequencies of maximum work output (~ *Y* ratio)

low levels of flightin accumulation show marked reductions in passive and dynamic stiffness, commensurate with an almost complete loss of stretch activation and power output. Notably, another myosin rod point mutant with reduced levels of phosphorylated flightin (*Mhc*⁶: Table 1) exhibits no significant reduction in dynamic elastic modulus, but does show a significant reduction in dynamic viscous modulus that accompanies a sharp drop in oscillatory power output (Henkin et al., 2004). This result raises the possibility that oscillatory power production is modulated by changes in flightin phosphorylation. However, one cannot exclude the possibility that the reduced power output in both *Mhc*¹ and *Mhc*⁶, listed as myosin rod mutations in Table 1, is partly due to a structural defect in the rod itself.

Actin and actin-associated proteins. Like the thick filament, various proteins that bind to filamentous actin reinforce the thin filament. It stands to reason that the longitudinal strength and stiffness of the thin filament lattice must be more or less equivalent to that of the thick filament lattice, else the thin filament would represent a weak link in the system. Although the thick filament is the focus of this review, it is important to mention those attributes of the thin filament lattice that allows it to match to the strength and stiffness of the thick filament. First, the ratio of thin to thick filaments is 3:1 in insect flight muscle, so each thin filament does not have to support as much tension as each thick filament. Second, the I-bands of the IFM sarcomere are very narrow (~0.5 μm), representing about 15% of the sarcomere length (~3.2 μm). A short, stiff connection from the ensemble of working cross-bridges to the Z-band (Fig. 1) provides a crucial link for effective power transmission.

Proteins associated with filamentous actin play an ancillary but key role in further stiffening the thin filament. In addition to its calcium-dependent regulatory role, **tropomyosin** (TM, Fig. 1) stiffens the thin filament stiffness by continuously running alongside the actin monomers, 'spot welded' to them via electrostatic interactions, within each groove of the double-stranded actin filament (Cammarato et al., 2004). *Drosophila Troponin H* (TnH, Fig. 1), unlike its *Lethocerus* counterpart that forms part of the troponin complex, is an elongated TM fusion protein that is roughly two-thirds longer than the standard TM, either lies alongside or periodically replaces TM at a molar ratio of ~ 1:1 (Clayton et al., 1998). IFM from mutants that lack the standard tropomyosin are structurally weaker, are less stiff, and produce significantly less power than wild type flies or flies rescued with the wild type standard TM gene (Kreuz et al., 1996; Miller et al., 1993; Molloy et al., 1993). IFM from mutants with reduced expression of TnH are also compromised structurally, producing less power than control flies, but the reduction is not nearly as severe as that of the TM mutant at comparable levels of expression (Kreuz et al., 1996). Ultrastructural analysis of TM and TnH mutants indicate both proteins are required for normal myofibril assembly.

Filamentous proteins of the supertitin family also contribute to the stiffness of the sarcomere (Fig. 1) via their interactions with the myofilaments. **Projectin** (Saide, 1981) and **kettin** (Kulke et al., 2001) link thick filaments to Z-disc proteins. Both contribute to passive stiffness (Kulke et al., 2001; Vigoreaux et al., 2000). Projectin, like titin, appears to be involved in enhancing stretch-activation (Granzier and Wang, 1993; Vigoreaux et al., 2000) and it is likely kettin is similarly involved (Kulke et al., 2001). Both bind to Z-disc proteins, and both probably share a close association with the thin filament in the I-band region before attaching to the thick filament as has been demonstrated for titin in vertebrate muscle

(Granzier et al., 1997). IFM from flies with a portion (~15%) of the wild type projectin replaced by a mutant C-terminally truncated form (*bent^{D/+}*; Table 1) show reduced oscillatory work output, consistent with a loss of contact with actin or myosin, resulting in a more compliant link to the power-producing actomyosin cross-bridges. But *bent^{D/+}* IFM also exhibit enhanced kinetics (an increase in frequency at which maximum work is generated), the net result of which is unchanged power output. Enhanced kinetics, which cannot be simply explained on the basis of increased thin filament compliance (Wang et al., 1999), is probably related to the removal of a putative kinase domain (Weitkamp et al., 1998) within the deleted sequence. The substrate may be RLC (Dickinson et al., 1997), flightin (Vigoreaux and Perry, 1994), troponin T (Domingo et al., 1998), troponin I (Weitkamp et al., 1998), or projectin itself (Maroto et al., 1992). A change in the phosphorylation state of any one of these possible substrates of projectin kinase may affect the contractile properties of the IFM.

4. SUMMARY AND CONCLUSION

Table 1 summarizes the primary mechanisms most likely responsible for modifying wing beat frequency (WBF) and muscle power in the *Drosophila* mutants discussed above. The different outcomes reflect different mechanisms that come into play, depending on the protein and site of the mutation. For example, the reduced muscle power and WBF of the RLC phosphorylation site mutant *Mlc2^{S66A,S67A}* reflect the reduced number of myosin heads available to form working cross-bridges and the concomitant reduction in muscle stiffness. The mixed results of the other mutants are more difficult to explain. For example, while the reduced muscle stiffness of the paramyosin rod mutant *pm^{S18A}* and the projectin mutant *bent^{D/+}* may in part reflect mutation-related increases in compliance of the thick filaments (*pm^{S18A}*) or connecting filaments (*bent^{D/+}*), the elevated WBF is unexpected because one would expect reduced muscle stiffness to lower WBF rather than raise it¹. Other aspects of the results are equally baffling. In the case of *pm^{S18A}*, e.g., myofilament kinetics are enhanced, opposite to what one would predict from reduced myofilament stiffness (Wang et al. 1999), but consistent with a direct effect of the mutation on cross-bridge kinetics. It is tempting to speculate that the fly increases the resonance frequency of its flight system, perhaps even over-compensating, as a mechanism for bringing the optimum frequency of power output of the flight system in line with the optimum frequency of power output of the myofilaments in order to achieve flight. The fly might accomplish this by voluntarily activating flight control muscles that change the stiffness and shape of the thoracic box (Tu and Dickinson, 1996), thereby significantly changing the basal stiffness of the resonance system. This effective

¹ The flight system of *D. melanogaster* is essentially a resonance system, whose fundamental frequency, in its simplest formulation, is directly proportional to the square root of the dynamic stiffness of the indirect flight muscles (Hyatt and Maughan, 1994; Molloy et al. 1993). Of course, other factors modify wing beat frequency, such as the length of the wings and their mass, as well as central nervous system recruitment of voluntary (synchronous) direct flight muscles (Lehmann and Gotz, 1996).

strategy would serve to tune flight system kinetics to that of the actomyosin motor for optimum power transmission.

Notably, of the four thick filament mutations listed in Table 1 produce no significant changes in wing beat frequency, three exhibit reduced muscle power, so these flies must make other adjustments to maintain flight competency. These may be additional cases in which the effects of marked changes in cross-bridge kinetics (MHC *IFI-EC*), cross-bridge deployment (*Mlc2^{A2-46}*), or sarcomere (thick filament) stiffness (*pm^{S-A4}* and *Df(3L)fln^{1/+}*) are ameliorated by the intervention of direct flight muscles. In summary, it may well be that the fly's general response to mutations that alter one component of the flight system is to alter another in order to maintain optimum transmission of power and flight competency. That is, nature's strategy for optimizing power generation throughout the flight system is probably the same as that at the level of the myofibril: that is, strengthen weak links, orient parts for optimum power production, and modify power train proteins through isoform switches or post-translational modifications to assure all components are in tune with one another.

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