muscles subjected to 25 hz stimulation (flight frequency) are partially fused with (likely) little crossbridge turnover. To assess the structural correlates of temperature gradients, we performed small-angle x-ray fiber diffraction measurements as a function of position along the dorsal-ventral thermal gradient in intact moth thoraces. The equatorial intensity ratio (I_{20}/I_{10}) in unstimulated muscle increased by ~25% in the first 1 −1.5 mm traversing from dorsal to ventral, implying that increased temperature was associated with increased association of the myosin heads with the thin filaments presumably predisposing them towards more productive acto-myosin interaction. Interestingly, X-ray patterns from skinned muscle preparations improved with increasing temperature indicating better structural order. Together, these observations suggest that cooler, superficial muscles may act mainly as elastic energy storage, whereas warmer deeper muscles may do the bulk of the mechanical work.

1809-Pos

Positioning of Myosin-Binding Protein C in Skeletal Muscle

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Frog striated muscle gives a pair of X-ray meridional reflections at spacings of ~419Å and ~442Å, which Offer (CSH Symp.37, 83-97, 1972) and Rome (ibid., 331-339) have shown are related to the disposition of C-protein in two sets of bands at ~430Å intervals on either side of the H-zone, giving rise to interference fringes that sample the underlying 430Å reflection. However, there are problems with this simple interpretation.

We have studied these reflections at high resolution on the BioCAT beam line at the Argonne National Lab., in both relaxed and contracting muscles, and during the onset of activation. In resting muscle, two main peaks can generally be seen in the relevant region, usually at ~419Å and ~442Å as previously described, but the latter peak is about 4 times more intense than the former, which would require an underlying sampled peak at ~437Å. It seems unlikely that the C-protein repeat is different from the helical repeat of the myosin filament to which it is attached (429.6Å), and more probable that some second component is involved, namely a ''forbidden'' first order myosin meridional reflection, as discussed by Malinchik and Lednev (JMRCM 13, 406-419, 1992), The interference fringes generated by this repeat would interact in a complex way with those from C-protein, since the reflections would in general have different phases. We find that the observed patterns, with very strong ~442Å reflections, can be modeled very satisfactorily even when both underlying repeats are kept at 429.6Å.

Passive stretch of semitendinosus muscles to sarcomere lengths up to the 3.2 - 3.5 μm range, where overlap between the C-protein bands and actin becomes zero, has little effect on the spacing of these reflections. However, that does not mean there is no interaction between C-protein and actin.

1810-Pos

Different Orientation of Two Heads of a Myosin Crossbridge in Full-Filament Overlapped and Overstretched Muscles Obtained by X-Ray Fiber Diffraction

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A novel method using the cylindrically averaged difference Patterson function was applied to correct a sampling effect due to the hexagonal filament array on the thick filament-based layer-line intensities from frog skeletal muscles at the full-filament overlap length. Using the corrected intensity data and the mixed structural model of a thick filament with two different axial periodicities of the myosin crossbridges, we performed an optimum search of azimuthal orientation of two heads of a myosin crossbridge and compared the optimum orientation to that from muscles stretched beyond filament overlap reported previously. The result showed that the myosin crossbriges in the regular repeating region had a similar configuration in both muscles. Two heads of a myosin crossbridge formed a windmill-shape when seen from the top of the filament and one head of a myosin crossbridge seemed to be almost in contact with another head in a pair at an adjacent crown level along the filament axis. One head was toward the converter domain of the other head, similar to regulated myosin heads in Tarantula muscles in which the intramolecular head-head interaction occurs. In the perturbed region, however, myosin crossbridges had different configurations in these muscles. In top view, two heads of a myosin crossbridge showed a U-shape structure in the overstreched muscles while a cross-shape structure in muscles with the full-filament overlap. One myosin head seemed to be in contact with the other head at the same axial crown level. The models suggest that the disposition of two-headed myosin crossbridges is stabilized by the head-head interaction at same or different axial crown levels. Probably this would be related to the inhibition mechanism of actomyosin interaction in the relaxed muscles.

1811-Pos

Relative Contribution of Attached and Detached Myosin Heads to the X-Ray Pattern from Skeletal Muscle

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In the X-ray diffraction pattern from skeletal muscle the third order myosin-based meridional reflection, M3, originates from the axial repeat of myosin heads along the thick filament. Changes in the intensity (I_{M3}), spacing (S_{M3}), and fine structure (R_{M3}) of the M3 reflection in contracting skeletal muscle at full filament overlap (~2.1 μm sarcomere length) have been measured in many different protocols (Piazzesi *et al. Nature***415**:659,2002; Reconditi *et al. Nature***428**:578**,** 2004; Linari *et al. J. Physiol.***567**:459, 2005; Huxley *et al. J. Mol. Biol.***363**:743, 2006; Huxley *et al. J. Mol. Biol.***363**:762, 2006; Brunello *et al. PNAS***104**:20114, 2007; Piazzesi *et al. Cell***131**:784, 2007). These studies showed the presence of a fixed periodic mass that is insensitive to filament sliding and attributed to detached myosin heads, but estimates of the relative contribution of the detached heads to the M3 reflection ranged from 0.3 to 0.6. Here we show that this parameter can be constrained by the dependence of the M3 reflection on sarcomere length (*sl*). When *sl* is increased from 2.1 to 3.20 μm, decreasing the fraction of myosin heads that are overlapped by actin filaments from 1 to 0.3 (and thus, according to Piazzesi *et al.* 2007, the fraction of actin-attached myosin heads from 0.3 to 0.09), force and I_{M3} decrease in proportion to filament overlap, while S_{M3} and R_{M3} are approximately constant (Linari *et al*. *PNAS***97**:7226, 2000). These results suggest that in isometric contraction at full filament overlap the contribution to I_{M3} of detached myosin heads is no more than 35% of that of attached heads and that there is very little axial offset between the two head populations.

1812-Pos

Myosin ATP Turnover Rate: a Mechanism Involved in Thermogenesis in-Resting Skeletal Muscle Fibers

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Thermogenesis by resting muscle varies with conditions and plays an active role in homeostasis of body weight. The low metabolic rate of living resting muscles requires that ATP turnover by myosin be inhibited relative to the purified protein in vivo. This inhibition has not been previously seen in in vitro systems. We used quantitative epifluorescence microscopy of fluorescent nucleotides to measure single molecule turnovers in relaxed permeable skeletal muscle fibers. We observed two lifetimes for nucleotide release by myosin: a fast component with a lifetime of 0.2-0.3 minutes, similar to that of purified myosin, and a slower component with a lifetime of 3.8+/− 0.4 minutes. We define the latter component to be the 'super relaxed state'. The fraction of myosins in the super relaxed state was decreased at lower temperatures, by substituting GTP for ATP or by increased levels of myosin phosphorylation. All of these conditions have also been shown to cause increased disorder in the structure of the thick filament. We propose a model in which the structure of the thick filament modulates the nucleotide turnover rates of myosin in relaxed fibers. Modulation of the relative populations of the super relaxed and conventional relaxed states would have a profound effect on muscle thermogenesis, with the capacity to significantly alter whole body metabolic rate. The mechanism proposed provides a new target for therapeutics with the potential to treat to obesity or help in controlling high blood sugar levels.

Muscle Regulation II

1813-Pos

A Disulfide Bond at Cys 190 of Tropomyosin Alters Tryptic Cleavage Pattern

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Native tropomyosin (Tm), an α-helical coiled-coil, possesses a charged acidic amino acid (Asp 137) that occurs in a hydrophobic position which destabilizes the coiled-coil. This region is sensitive to tryptic cleavage and is important in the proper regulation of the myosin activate ATPase, (Sumida, John P., Wu, Eleanor, Lehrer, Sherwin S, JBC 283, 2008). Thermal stability measurements of Tm suggest a long-range interaction between the Asp 137 position and the Cys 190 position. In the current work, we present further evidence of long-range interactions along the length of tropomyosin.

Native frog Tm was cross-linked with DTNB at Cys 190. Tryptic cleavage patterns of this cross-linked species were compared to a control Tm in which the Cys 190 groups were maintained reduced state using DTT. The course of tryptic cleavage of the cross-linked molecule was altered compared to the fully reduced molecule. Proteolysis of the cross-linked molecule produced new tryptic fragments seen in a reduced SDS gel at 30kDa and 20kDa. Trypsin cleaves reduced tropomyosin at Arg 133 to produce two new tryptic fragments (17kDa and 15kDa). The observation that cross-linked Tm exhibits a change in the cleavage rate, and affects the cleavage pattern, demonstrates a long-range effect of the cross-link at Cys 190 on the site of trypsin cleavage, Arg 133. This finding adds evidence to the idea that ''flexibility'' is associated with a locally fluctuating region in the middle of the molecule which has implications on our understanding of how Tm moves on the actin surface, and provides insight into understanding how single point mutations in Tm may result in dysfunction and disease associated with various myopathies.

1814-Pos

Tropomyosin Pseudo-Phosphorylation and Muscle Kinetics

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Tropomyosin contains a phosphorylation site at Ser-283 located within the head-to-tail overlap region that regulates muscle contraction. Previously we demonstrated that recombinant wild-type (Tm-WT) and pseudo-phosphorylated (Tm-S283D) alpha tropomyosin, expressed and purified from insect cells, exhibit regulated ATPase activity and troponin T binding similar to that reported for non-phosphorylated and phosphorylated tropomyosin (pTm) purified from muscle. We further demonstrated transgenic mice expressing the pseudo-phosphorylated tropomyosin (Tm-S283D Tg) exhibit increased mortality and decreased rate of relaxation in the work performing heart preparation. These changes occur in the absence of altered steady state maximal force or calcium-sensitive force development in skinned papillary bundles. In light of these findings, we sought to investigate the effect of pTm on the kinetics of cardiac muscle function. Using an extraction/replacement protocol, we measured force kinetics in a myofibril preparation. Results demonstrate no significant difference between myofibrils replaced with Tm-S283D or Tm-WT in maximal force, the rate of force activation, or the rate of force redevelopment, implying pTm does not play a role in altering muscle activation or cycling kinetics. To investigate if pTm affects the kinetics of thin filament inactivation we measured the rate of calcium disassociation from troponin C. Results demonstrate thin filaments reconstituted with Tm-S283D decreased the rate of calcium disassociation from troponin C consistent with the previously observed relaxation impairment. Finally, to determine the effects of pTm on systemic alterations in cardiovascular performance we measured heart function in Tm-S283D Tg mice by echocardiography. Results demonstrate Tm-S283D Tg mice exhibit trends towards impaired cardiac contractility compared to non-transgenic mice including decreased peak systolic velocity and ejection fraction. Overall, these findings demonstrate tropomyosin phosphorylation contributes to the regulation of cardiac dynamics; however, the precise role of pTm in the development of cardiac dysfunction remains elusive.

1815-Pos

Studies of the Mid Region of Tropomyosin Using an Incorporated Tryptophan Analogue

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Striated muscle contraction is controlled by highly sensitive alterations in thin filament conformation that involve a Ca2+-dependent interaction between troponin I (TnI) and actin-tropomyosin (Tm). To study these interactions, 5-OH Tryptophan (OHW) was incorporated into striated muscle alpha-tropomyosin. A Single Trp tropomyosin mutant, Q135W, was generated and expressed in Trp auxotrophs. OHW incorporation was 76%. Thin filaments containing Q135W retained excellent function: 12-fold Ca2+ regulation in myosin S1-thin filament MgATPase assays. Fluorescence emission titrations with increasing amounts of Ca2+ were performed (emission max 338nm) in presence of thin filaments comprised of cardiac troponin, F-actin, and Q135W tropomyosin. Similar titrations using troponin, actin, and wt Trp-free tropomyosin produced low fluorescence (ex wavelength 312 nm). Ca2+ decreased the OHW fluorescence of Q135W-containing thin filaments by 7.0 +/− 0.4 %. This transition occurred with K = 1.8 +/− 0.3 uM −1, consistent with the affinity of the cardiac thin filament regulatory calcium site on TnC. OHW Q135W tropomyosin is a novel tool for monitoring thin filament behavior, in particular for detecting Ca2+ binding to troponin. Troponin has been viewed as binding to the C-terminus of tropomyosin, which does not include the mid-tropomyosin residue 135 that is found in the current study to be Ca2+-sensitive. However, the findings may be related to recent results, suggesting that the TnI C-terminus of troponin bridges from one actin strand to the other, and contacts tropomyosin near residue 146. Galinska-Rakoczy et al, JMB 2008. Mudalige et al, JMB2009. Finally, human cardiac troponins with defective inhibitory activity due to alterations in the TnI inhibitory region have been generated. They are currently under study using this tropomyosin.

1816-Pos

Loss of Function in β-Tropomyosin (TPM2) Mutants Causing Nemaline Myopathy or Cap Disease

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Nemaline myopathy (NM) and cap disease (CD) are two congenital skeletal muscle myopathies characterized by general muscle weakness with early neonatal onset. They are both clinically and genetically heterogeneous, but at the histological level they present characteristic pathology: nemaline rod-like bodies within myofibers generating from the Z-discs for NM and cap-like structures at cell periphery underneath the sarcolemma for CD. Recently several different mutations on the TPM2 gene, coding for the -tropomyosin (-Tm) isoform expressed in skeletal muscles (especially slow-twitch type 1 fibers), have been reported to cause either NM or CD. In this study six -Tm mutants connected either to NM (K7, E117K, Q147P) or to CD (E41K, K49, E139) and the wild type form have been expressed in a baculovirus/sf9 system. This system, being based on eukaryotic cell cultures, permits the N-terminal acetylation of -Tm, which has been shown to be important for its linear head-to-tail polymerisation. Thin filaments reconstituted with these recombinant proteins have been tested with the *in vitro* motility assay finding a considerable loss of function for each of them. K7, Q147P, K49 and E139 showed, through indirect observations, a reduced affinity to actin, possibly explained by destabilising the Tm homodimer itself or important actin-Tm binding sites. With all mutants the Ca^{2+}-induced increase in sliding speed was greatly reduced, indicating an altered TroponinT-Tm interaction, but thin filaments Ca^{2+} switching was unaltered. The three NM mutants showed a reduced Ca^{2+}-sensitivity, whereas two of the CD mutants showed an increase. E41K had a different phenotype and seemed to strongly inhibit filament motility. A well-defined scenario is taking shape and further studies could directly correlate these findings with the etiology of the diseases.

1817-Pos

An Evolutionary Structural Bioinformatics Analysis of the Actin Binding Protein, Tropomyosin

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Tropomyosin is a two-chained, α-helical coiled-coil protein that associates end-to-end to form a continuous strand along actin filaments and regulates the functions and stability of actin. Mutations in tropomyosin cause skeletal and cardiomyopathies. We have carried out a phylogenetic analysis of tropomyosin to identify its conserved residues and to elucidate their importance for binding actin. Actin is a highly conserved protein and our hypothesis is that the actin binding sites of tropomyosin have been conserved through evolution to retain its actin-binding function. Phylogenetic trees of 60 coding sequences were constructed from tropomyosin genes from 19 species within the phyla Chordata, Hemichordata and Echinoderm. The rates of substitution (ω) at amino acid sites (codons) in the protein sequence were calculated to identify the most conserved sites (lowest ω) using CODEML in PAML 4.1. A total of 103 out of 284 residues were identified as highly conserved ($\omega \leq 0.015$), of which 24 are in *a* and *d* positions of the heptad repeat involved in the hydrophobic coiled coil interface, 38 are in potential interhelical *e-g* position salt bridges important for folding of the coiled coil, and 41 are in *b*, *c*, or *f* surface positions available for binding to other proteins, such as actin. The conserved residues at the *b*, *c*, and *f* surface positions were selected for initial mutagenesis studies with mutations to alanine. Preliminary actin binding co-sedimentation assays carried out for three tropomyosin mutants showed a 2- to 3-fold decrease in actin-binding affinity compared to the wild-type protein. Further analysis of mutations at other conserved surface positions and their effect on actin-binding affinity and stability of the mutant proteins are in progress. *Supported by Muscle Dystrophy Association.*