exchange of S456 by a large amino acid would prevent the complete closing of switch-2 and therefore the full generation of the power stroke providing an explanation for the observed kinetic and mechanical defects of the mutant myosin. We have solved the crystal structure of the myosin motor domain with S456Y mutation in complex with ADP-VO_4. The overall crystal structure and conformation of the nucleotide binding region resembles that of the wild-type revealing that switch-2 indeed can adopt the 'closed' conformation. We therefore conclude that not the complete 'closing' but the complete 'opening' of switch-2 is required for the full power stroke. Additional conformational changes in the crystal structure of S456Y, e.g. the actin binding loop-2 and loop-4, explain the disturbed actin binding properties of the mutant construct.

742-Pos

A Single Amino Acid Mutation in the Drosophila Myosin SH1 Domain Severely Affects Muscle Function, Myofibril Structure, Myosin Enzymatic Activity, and Actin Sliding Velocity

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Hereditary inclusion-body myopathy type III (IBM-3) is caused by a single amino acid Glu706Lys substitution in the SH1 helix of the myosin head. The SH1 domain has been proposed to play a key role in the conformational changes that occur in the myosin head during force generation which is coupled to ATP hydrolysis. We are using an integrative approach to study the structure-function relationship of the myosin SH1 domain in the *Drosophila* model system. We constructed a gene encoding myosin with the single amino acid mutation and expressed it in place of wild-type myosin heavy chain by germline transformation and crossing into a line that lacks myosin in its flight and jump muscles. The homozygous flies are flightless and their jump abilities are also greatly reduced. The indirect flight muscle fibers of young flies show considerable ultrastructural disarray, with some regions of missing thick and thin filaments, and myofibrils that are not uniform in width. Our initial study showed that actin sliding velocity and basal and actin stimulated ATPase were reduced more than 70% compared to wild-type indirect flight muscle myosin. Homology models indicate that the surface charge change of the substitution in the highly conversed SH1 region could destabilize the helix, which is critical for the converter domain to rotate to its full range of movement during the power-stroke. This structural change would affect the lever arm swing, resulting in dysfunctional myosin. Given that human IBM-3 is mild in childhood but severe during aging, with the accumulation of inclusion bodies, we are investigating whether inclusion bodies or aggregates appear in aged mutant *Drosophila* muscle tissues.

743-Pos

FRET To Reveal Cross-Bridge Conformational Changes

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Myosin is an actin-based motor protein generating force through ATP hydrolysis. Cross-bridges reversibly bind to actin producing sliding of the myofilaments by cycling between actin-attached (strong binding: ADP or rigor) and actin-detached (weak binding: ATP or ADP-Pi) states. The myosin and actomyosin ATPase mechanisms have been intensively studied [1], however, the specific conformational changes that take place and their link to ADP, Pi release, production of mechanical impulse and the consequent muscle contraction remain unclear. In this work we exploit FRET (Forster Resonance Energy Transfer) as an assay to monitor the dynamics of cross-bridge conformational changes directly in single contracting muscle fibres. The advantage of FRET-imaging in order to reveal such movements is related to its ability to measure distances in the nm range, relevant for structural changes in actomyosin cross-bridges [2]. To reach this goal we use several FRET pairs to investigate different locations in the actomyosin complex. In particular, a genetically modified essential light chain bearing a single cysteine residue at position 178 labelled with different thiol-reactive chromophores (Alexa488 or 5-IAF, being donor or acceptor) has been exchanged with native light chains of myosin into permeabilised muscle fibres[3]. The other fluorophore has been introduced by either labelling actin filaments (rhodamine phalloidin as acceptor for Alexa488), SH1 cysteine (Rhodamine, as acceptor) or the nucleotide binding site with an ATP-analogue (DEAC-pda-ATP, as donor for 5-IAF)[4]. Preliminary experimental data show FRET signals in muscle fibres, indicating the viability of the approach to reveal structural changes at the cross-bridge level.

[1] Geeves, M.A. and K.C. Holmes. Advances in Protein Chemistry 2005
[2] M.Sun et al. Pnas 2008
[3] J.Borejdo et al. Biochemistry 2001
[4] D.I. Garcia et al. Biophys J. 2007

744-Pos

Electron Microscopic Evidence for the Cross-Bridge Lever Arm Mechanism in Living Muscle Thick Filaments Obtained using the Gas Environmental Chamber

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We have succeeded in recording the ATP-induced cross-bridge recovery stroke in living bipolar muscle thick filaments using the gas environmental chamber (EC) (Sugi et al., PNAS 105:17396, 2008). It is generally believed that the distal part of the cross-bridge (catalytic domain) is rigid, while its proximal part acts as a lever arm moving around the hinge to produce force and motion in muscle. To ascertain the validity of this mechanism by our experimental methods, we prepared three different antibodies, directed to the peptide in the cross-bridge catalytic domain (antibodiy 1), to the reactive lysine residue at interface between the catalytic and lever arm domains (antibody 2), and to the peptide in the cross-bridge lever arm domain (antibody 3), respectively. These antibodies, attached to the cross-bridges on the thick filaments, were position-marked with colloidal gold particles.

The peak amplitude of the ATP-induced movement of the cross-bridges with antibody 1 (5~7.5nm) did not differ significantly from that of the cross-bridges with antibody 2, being consistent with the idea that the cross-bridge catalytic domain remains rigid during the cross-bridge stroke. On the other hand, the amplitude of the ATP-induced movement in the cross-bridges with antibody 3 was found to be extremely small and in most cases just barely detectable (2.5nm or less), indicating that the proximal part of the cross-bridge (close to the lever arm hinge region) does not move appreciably during the ATP-induced cross-bridge stroke. These results may constitute the first direct evidence for the cross-bridge lever arm mechanism in muscle contraction.

745-Pos

Comparative Kinetics of the ATPase and Actin Sliding Velocity of Myosin Isoforms

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Myosin isoform expression varies according to demand and pathology, and the kinetics of the resultant actomyosin motor protein determine maximal sarcomere shortening velocity. Studies of muscle fibers and isolated myosin isoforms have shown that actin sliding velocity correlates with ATP hydrolysis. We studied this relationship for isoforms of actomyosin complexes and examined ATP hydrolysis and the effect of association and dissociation of myosin with actin. Sliding velocity of actin filaments was measured in motility assays with different myosin isoforms. Actin-dependent ATP hydrolysis rate of isolated myosin sub-fragments interacting with filamentous actin, or cross-linked with actin by the zero-length cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were measured. ATPase activity and velocity of actin moved by myosin isoforms from rat and canine cardiac and skeletal muscle were measured at 25°, 30° and 35°C. Motility velocity plotted against ATPase activity of different myosin isoforms showed a linear correlation with a slope of 330 ± 20 (nm/sec)/(ATP/sec) ($R^2 = 0.98$); the slope coefficient was 19% of the slope of the relationship for intact muscle described by M. Barany (J Gen Physiol, 1967) across a wide range of temperatures. Activation energy of sliding velocity (92 - 96 kJ/mol) and ATPase rate (83 - 121 kJ/mol) of different myosin isoforms were similar. Cross-linking of actomyosin complexes by EDC increased ATP hydrolysis rate 4-fold above ATPase at saturating [Actin]. This suggests that association/dissociation kinetics are rate-limiting and that ATPase is activated in maximally 25% myosin molecules interacting with actin in solution. The calculated displacement of actin filaments ($D = 330$ nm) per ATP hydrolyzed under the experimental conditions used here suggests that unloaded cross-bridges may displace actin over a multitude of the minimal steps of 2.7 nm that can be made by myosin along the actin filaments.

746-Pos

Correlation between Myofibrillar Biochemistry and Muscle Fiber Mechanics using Rabbit Psoas Muscle Preparations Indicates that Phase 2 of Step Analysis Represents the Cross-Bridge Detachment Step

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Our goal is to correlate results obtained from myofibrillar suspensions and muscle fibers. For myofibrils, tryptophan fluorescence with stopped-flow apparatus was used; for fibers, tension transients with small amplitude sinusoidal length

perturbations were used. Experiments were performed in identical solutions with 0.2M ionic strength at pH 7.00. The concentration of MgATP was varied to detect kinetic constants of the ATP binding step 1 (K_1: dissociation constant), the cross-bridge detachment step 2 (k_2, k_{-2}: rate constants), and the ATP cleavage step 3 (k_3, k_{-3}). By following the fast rate constant at 20°C, we found in myofibrils: k_2/K_1=1.0∈ $\mu M^{-1}s^{-1}$, K_1=0.3mM, k_2=300s^{-1}, and $k_{-2} \approx 0$; in fibers: k_2/K_1=0.23∈ $\mu M^{-1}s^{-1}$, K_1=1.58mM, k_2=363s^{-1}, k_{-2}=180s^{-1}. From these results we conclude that (1) ATP binding is ~5X stronger in myofibrils than in fibers, (2) cross-bridge detachment rate is just about the same, and (3) its reversal step is almost absent in myofibrils, but it is finite in fibers. Consequently, we found a good agreement in the results obtained from myofibrils and fibers, indicating that phase 2 of tension transients from step analysis in fibers (Huxley and Simmons, 1971) represents the cross-bridge detachment step. We also studied actin-myosin cross-linked myofibrils and found no difference, indicating that cross-linking does not significantly modify steps 1-3 kinetics. We further studied the Pi effect in myofibrils, and found that Pi is a competitive inhibitor of MgATP with the inhibitory dissociation constant of 7-8mM. To deduce the kinetic constants of the ATP cleavage step, we measured the slower rate constant in fluorescence in myofibrils and found that k_3+k_{-3}=10.7s^{-1} at 4°C. From the Pi burst experiments using radioactive ATP, we found that K_3=6.1 at 4°C. From these, k_3=9.2s^{-1} and k_{-3}=1.50s^{-1} were deduced.

747-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 vitro. This inhibition has not been previously seen in in vitro systems. We used quantitative epifluorescence microscopy of fluorescent nucleotides to measure single nucleotide 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 obesity or help in controlling high blood sugar levels.

748-Pos

Structural Impact Of Myosin Methionine Oxidation

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We have examined the structural and functional consequences of methionine (Met) oxidation in Dictyostelium (Dicty) myosin II using a three-pronged approach that includes Met mutagenesis, site-directed spectroscopy, and molecular dynamics simulations. Protein oxidation by reactive oxygen species (ROS) is a critical element of cell function, but in the context of oxidative stress, has been implicated in disease progression and biological aging. Our goal is to bridge our understanding of protein oxidation and muscle dysfunction with molecular-level insights into actomyosin interaction. A Cys-lite version of Dicty myosin II serves as our model system for examining site-specific Met oxidation. Peroxide treatment to mimic oxidative stress induced a two-fold decline in Vmax and KATPase for actin-activation, consistent with the decline in actomyosin interaction observed for biologically aged or peroxide-treated skeletal myosin. We tested the oxidation sensitivity of previously characterized myosin labeling sites in the force-producing region and actin-binding interface and found that spin label mobility and distance measurements in the actin-binding cleft are particularly sensitive to Met oxidation, but only in the presence of actin. Moreover, we conclude that the oxidation-induced structural change in myosin includes a redistribution of structural states involved in the weak to strong actin-binding transition, the step associated with muscle force production. Site-specific Met substitutions combined with functional measurements have allowed us to pinpoint which Met is responsible for the observed structural change. Lastly, we will examine Met oxidation in silico to gain mechanistic knowledge of how residue-specific oxidation translates into changes in both local and global myosin structural dynamics. We expect that our results will be applicable to the many biological and pharmaceutical contexts in which a detailed understanding of protein oxidation, function and structure relationships are sought. This work is supported by the NIH training grant ''Functional Proteomics of Aging'' (T32AG029796).

749-Pos

Novel Approach Applied to IVMA to Study the Modulation of the Actomyosin Interaction by MgATP In Fast Skeletal Muscle

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In this study we used an ''in vitro motility assay'' (IVMA) approach to investigate the effect of the variation of [MgATP] in determining the number and the sliding velocity (Vf) of actin filaments moved by fast skeletal myosin. Vf was studied on type 2B HMM from rats at 25C°, 100mM ionic strength and at varius [MgATP], [MgADP] and [Pi]. We designed a new experimental set-up to perform experiments at lower ionic strength and in buffers that had no interference with the ionic environment, in order to mimic physiological condition. This set-up allowed a complete and chemical speciation of the solutions opening the possibility to perform an accurate thermodynamic study. Therefore, along with kinetic measurements also quantitative thermodynamics measurements were carried obtaining the $\in''G$ of MgATP hydrolysistaking into account pH and [Mg^{2+}]. We correlate the thermodynamics property of the system to Vf and to the number of sliding actin filaments which were assessed by a purpose-designed software. Preliminary results indicate: **a)** no straightly correlation between values of $\in''G_{ATP}$and the velocity of actin filaments, **b)** an increase in thenumberof sliding actin filaments at low [MgATP] and no changes when the ratio [MgATP] / [HMM] was kept constant **c)** a decrease in the velocity of actin filaments at [Pi]=30mM**.** More investigations are required to confirm the unexpected results that indicate a complex role of MgATP and its metabolites in the modulation of actomyosin interaction.

750-Pos

The Effects of Head-Head Interactions on Myosin-Based Actin Sliding Velocities

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Myosin generates force with its weak-to-strong actin binding transition and senses force through a strain-dependent step closely associated with ADP release. We have developed both analytical and computational models of the cooperative interplay between these force-generating and force-sensing biochemical transitions. These models make several novel predictions for unloaded shortening muscle, such as rate constants, k_D, for ADP release that are [ADP]- and [ATP]-dependent. The model also predicts that the acceleration of k_{-D} is associated with a dissipation of interhead strain. To test these model predictions, we use an in vitro motility assay to determine the effects of [ADP] and [ATP] on actin sliding velocities, V, and to determine changes in interhead strain by measuring the rate of actin filament breaking. Our results show a non-hyperbolic nucleotide-dependence of V and a nucleotide-dependence of the rate of actin filament breaking that are both consistent with our cooperative model.

751-Pos

Single Molecule Stepping and Structural Dynamics of Myosin X

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Myosin X is an unconventional myosin motor protein with puzzling motility properties that are under debate. We studied the motility, angular motions and stepping of myosin X in vitro using single molecule fluorescence of rhodamine bound to the lever arm calmodulins (CaMs) and quantum dots on the CaMs and at the C-terminus of the heavy chain. Myosin X walks processively both on single actin filaments and actin bundles. The average step size, measured by FIONA, is 34 nm, supporting the postulate that an α-helical domain extends the lever arm beyond the binding region of myosin X for its three CaMs per head. The step size and velocity are smaller on actin bundles than individual filaments, suggesting that myosin X often steps onto neighboring actins in a bundle. Alternating larger and smaller steps with FIONA and alternating axial angles of the lever arm measured with polTIRF imply that myosin X steps in a hand-over-hand manner. Single molecule 3-dimensional (3D) tracking by Parallax of quantum dot-labeled myosin X on actin filaments and bundles suspended above the coverslip, by flow over ridges or by dielectrophoresis