dye-labeled lipid moieties will bring new insights into lipid organisation and trafficking at the synapse.

## Muscle: Fiber & Molecular Mechanics & Structure I

**737-Pos**

**Myosin Nucleotide Pocket Thermodynamics Measured by Epr Reveal How Energy Partitioning Relates Speed to Efficiency**

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We have used spin labeled ADP to investigate the dynamics of the nucleotide-binding pocket in myosins. In actomyosin•ADP the nucleotide-binding pocket is in an equilibrium between closed and open conformations, with the open conformation favored in slow myosins. In rabbit slow skeletal muscle fibers, the $\in''G$ for the closed to open equilibrium is $-3.9$ kJ mol$^{-1}$. We found similar values for pig ventricle myosin, chicken gizzard smooth muscle myosin, and chicken myosin V. For faster myosins, the equilibrium shifts to favor a closed conformation, rising to $-2.7$ kJ mol$^{-1}$ for *Dictyostelium discoideum* myosin II, $-1.9$ kJ mol$^{-1}$ for pig atrial myosin, $-1.1$ kJ mol$^{-1}$ for rabbit fast skeletal muscle fibers, and $+2.9$ kJ mol$^{-1}$ for *Drosophila* fight muscle fibers. We believe this represents a destabilization of the open actomyosin•ADP state in the faster myosins, driving ADP release. van't Hoff analysis of the temperature dependence of this equilibrium reveals that the closed to open conformation has a significant positive enthalpy and entropy, with $\in''H$ and $T\in''S$ of 40-50 kJ mol$^{-1}$ for slow myosins. Both components are reduced in this equilibrium for faster myosins, decreasing to $\in''H = 17.7$ kJ mol$^{-1}$ and $T\in''S$ at 25°C $= 18.8$ kJ mol$^{-1}$ for rabbit fast skeletal fibers, and $\in''H = 10.4$ kJ mol$^{-1}$ and $T\in''S$ at 25°C $= 7.5$ kJ mol$^{-1}$. Our model is that the open actomyosin•ADP state represents a partitioning point between the free energy released during the myosin catalytic cycle. Because of this partitioning, fast myosins destabilize the actomyosin•ADP state, reducing the energy available to do work up until that point, but leaving more free energy in reserve to drive ADP release. This gives a mechanism for the correlation between increased speed and reduced efficiency in muscle.

**738-Pos**

**Structural Basis for Uncoupling of Force Generation in the F506A *Dictyostelium* Myosin Revealed by Time-Resolved EPR and FRET**

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We have used dipolar electron-electron resonance (DEER) and time-resolved fluorescence resonance energy transfer (TR-FRET) to investigate the role of the myosin relay helix in coupling between the active site and the force-generating region of myosin II. Two double-Cys *Dictyostelium* myosin constructs have been engineered, and the structure of the relay helix was monitored by measuring interprobe distances in MSL/MSL or IAEDANS/Dabcyl-labeled myosin. Experiments were performed on WT myosin and on F506A, a functional mutant that has close to normal enzymatic activity but completely lacks motor functions (e.g. unable to move actin filaments or support cell development). We found that the WT myosin relay helix adopts two distinct states (straight and bent), with the bent conformation populated when ATP and ADP.$P_i$ analogs are bound at the active site. In contrast, binding of nucleotide analogs had very little effect on relay helix conformation in the F506A mutant. In addition, the width of the distance distribution was significantly larger in the F506A compared to WT myosin, indicating loss of structural organization. Our results demonstrate that the relay helix plays a key role in coupling of myosin ATPase and motor activities. Loss of functionality observed in F506A myosin can be explained by the disruption of the relay helix-relay loop interactions that normally stabilize well-defined conformations of the myosin force-generating region allowing it to switch between distinct structural states.

**739-Pos**

**Subpopulation of Intermediates in Actomyosin Crossbridge-Cycle During Sliding**

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We have been investigating conformational changes of myosin crossbridges during *in vitro* sliding, utilizing quick-freeze-replication and a novel image analysis to quantitatively compare microscopic images with the atomic models of various conformations. We reported that the leverarm moiety of working crossbridges, is mostly kinked to the opposite side of ADP/Vi-bound structure and such global configuration of myosin head resembles that whose SH1 and $-2$ are chemically crosslinked. Since the crystal structure of such unusual configuration is not determined, we attempted to reconstruct its 3-D structure by a special version of Single-Particle-Analysis, devised to adapt small-sized particle, utilizing very contrasty feature of metal-replicated images. With a new procedure to manage very few view-angles, we finally obtained the 3-D envelope of the myosin head with oppositely kinked leverarm, analogous to the intermediate structure under sliding conditions. We cut the original atomic model of pPDM-treated-ADP-structure (1L20) into motor-domain and the leverarm, and relocated each module at best-matching position and the orientation, to generate a tentative model that best-fits to reconstructed envelope. We then examined whether all the images of actin-sliding crossbriges can be uniquely explained by that standard SH-crosslinked structure model. The images of actin-attached crossbridges were classified by ''2-D appearance'' and each class-average was compared with 2-D projections of the standard structure as above. By analyzing the orientation of motor-domain and leverarm separately, we found that there could be several sub-populations, some matched to but some deviated from the standard structure. It is known that two reactive thiols can be crosslinked by a variety of bifunctional reagents of different span lengths, implying the distance between them might be flexible. We assume that the extension of new oppositely-kinked configuration would comprise the power-stroke and those sub-populations might correspond to several steps during that structural change.

**740-Pos**

**Analysis of Conformation of the Skeletal Muscle Myosin Modified by $F_2$DNB Using FRET**

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Previously biochemical studies have demonstrated that the highly reactive cysteine residues SH1 and SH2 can be crosslinked by variety of bifunctional reagents with different spans (3-14 Å) in the presence of nucleotides, suggesting that the region is highly flexible. The SH1-SH2 region is believed to play a key role in the conformational changes that occur in the myosin head during the force generation coupled to ATP hydrolysis. We have previously shown that the skeletal muscle heavy mero-myosin (HMM), which SH1-SH2 was crosslinked by 1,5-difluoro-2, 4-dinitrobenzene ($F_2$DNB) in the presence of ADP, have a novel conformation using quick freeze deep etch electron microscopy (QFDE-EM). We have also demonstrated that conformational change of the myosin motor domain during ATP hydrolysis can be monitored by measuring the FRET using fluorescent ATP analogue NBD-ATP. In the present study, we analyzed the conformation of the myosin crosslinked by $F_2$DNB using FRET between the ATP binding site and the essential light chain (ELC) A1. We prepared skeletal muscle myosin subfragment-1 (S1), which ELC was labeled by 6-bromoacetyl-2-dimethylaminonaphthalene (BD) at the Cys 177. And fluorescent ADP analogue NBD-ADP was trapped within the ATPase site of S1 labeled by BD. The FRET efficiency was estimated by measuring the change of fluorescence intensity of BD comparing with control BD-S1. The FRET efficiency of $F_2$DNB-S1-NBD-ADP was lower than S1-NBD-ADP state. This suggests that the $F_2$DNB-S1-ADP states form more kinked conformation than S1-ADP state.

**741-Pos**

**Switch-2 Dependent Modulation of the Myosin Power Stroke**

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Central to the mechanism of myosins are two conserved sequence motifs, switch-1 and switch-2, which contact nucleotide and $Mg^{2+}$ at the rear of the nucleotide-binding pocket. They act as gamma-phosphate sensors, thus controlling hydrolysis and product release. The movement of switch-2 from a 'closed' to an 'open' conformation is translated via the relay region into a rotation of the converter domain, which drives the force generating power stroke. To investigate the molecular details of this coupling mechanism, we introduced mutation S456Y in switch-2 of the myosin-2 motor domain from *Dictyostelium discoideum* and analyzed the structural and functional consequences. Our kinetic results showed that the S456Y mutant lost the ability to bind effectively ATP, displayed a strongly decreased actin affinity in the ADP-bound state, and moved actin filaments with highly reduced velocities. It has been proposed that the

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.

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**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 $330 \pm 20$ (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