

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

Muscle: Fiber & Molecular Mechanics & Structure I

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

Thomas J. Purcell¹, Nariman Naber¹, Kathleen Franks-Skiba¹, Ed Pate², Roger Cooke¹.

¹University of California, San Francisco, San Francisco, CA, USA,

²Washington State University, Pullman, WA, USA.

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 $\epsilon^{\circ}G$ 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 $+2.9 \text{ kJ mol}^{-1}$ for *Drosophila* flight 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 $\epsilon^{\circ}H$ and $T\epsilon^{\circ}S$ of 40-50 kJ mol^{-1} for slow myosins. Both components are reduced in this equilibrium for faster myosins, decreasing to $\epsilon^{\circ}H = 17.7 \text{ kJ mol}^{-1}$ and $T\epsilon^{\circ}S$ at 25°C = 18.8 kJ mol^{-1} for rabbit fast skeletal fibers, and $\epsilon^{\circ}H = 10.4 \text{ kJ mol}^{-1}$ and $T\epsilon^{\circ}S$ 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.

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

Roman V. Agafonov¹, Igor V. Negrashov¹, Sarah E. Blakely¹, Margaret A. Titus¹, Yuri E. Nesmelov², David D. Thomas¹.

¹University of Minnesota, Minneapolis, MN, USA, ²University of North Carolina, Charlotte, NC, USA.

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.

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Subpopulation of Intermediates in Actomyosin Crossbridge-Cycle During Sliding

Eisaku Katayama¹, Yoshitaka Kimori², Norio Baba³.

¹Institute of Medical Science, The University of Tokyo, Tokyo, Japan, ²Dept of Ultrastr. Res, Nat'l Inst. of Neuro.& Psychi., Kodaira, Tokyo, Japan, ³Dept of Inform. Sci., Kogakuin University, Hachioji, Tokyo, Japan.

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 crossbridges 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 cross-linked 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.

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Analysis of Conformation of the Skeletal Muscle Myosin Modified by F₂DNB Using FRET

Masafumi D. Yamada¹, Koichiro Ishiyama¹, Eisaku Katayama², Yoshitaka Kimori³, Shinsaku Maruta¹.

¹Dept. of Eng., Soka Univ., Hachioji, Tokyo, Japan, ²Div. of Biomol. Imag., Inst. of Med. Sci., The Univ. of Tokyo, Shiroganedai, Tokyo, Japan, ³Dept. of Ultrastr. Res., Nat'l Inst. of Neurosci., Nat'l Ctr. of Neurol. and Psych., Kodaira, Tokyo, Japan.

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 cross-linked by 1,5-difluoro-2, 4-dinitrobenzene (F₂DNB) 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 F₂DNB 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 F₂DNB-S1-NBD-ADP was lower than S1-NBD-ADP state. This suggests that the F₂DNB-S1-ADP states form more kinked conformation than S1-ADP state.

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Switch-2 Dependent Modulation of the Myosin Power Stroke

Daniela Kathmann, Ralph P. Diensthuber, Falk K. Hartmann, Roman Fedorov, Dietmar J. Manstein, Georgios Tsiavalariis. Medical School Hannover, Hannover, Germany.

Central to the mechanism of myosins are two conserved sequence motifs, switch-1 and switch-2, which contact nucleotide and Mg²⁺ 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