populations of photophysical states in the sample ensemble can be isolated by analyzing the time resolved decay. In a typical experiment, a probe (or pair of probes in the case of a FRET experiment) are innocuously attached to specific sites on myosin and actin. We then measure underlying protein dynamics by time resolved anisotropy and accessibility which reflect the local restricted motion of the probe, and by FRET which reflects the distance (or when $\kappa^2$ does not = 2/3, distance and the relative dipole orientation) between two probe pairs. When these measurements are performed during a biochemical transient initiated by stopped flow, we gain incite into the millisecond scale "structural kinetics" associated with the transition. These studies are made possible by the recent development of a high throughput time resolved fluorescence spectrometer which can measure a complete, high resolution, time resolved fluorescence decay, in 0.1 ms. The high throughput character of this instrument allows us to measure changes in the time resolved fluorescence decay and thus structural dynamics of a probe during a stopped flow initiated biochemical transient. The added dimensionality afforded by time resolved fluorescence isolates discrete populations of structural states in the ensemble. It also increases the measurement precision. We are using this approach to measure millisecond scale structural transitions that occur in myosin during nucleotide binding, hydrolysis and product dissociation and during actin binding and detachment.

**701-Pos Board B501**

**Orientation of the Myosin Regulatory Light Chain in Cardiac Muscle Determined by Polarized Fluorescence**

**Thomas Kampourakis**, Yin-Biao Sun, Andrea Knowles, Birgit Brandmeier, Mathias Gautel, Malcolm Irving.

The myosin regulatory light chain (RLC) is a component of the myosin lever arm, and the cardiac RLC isoforms are common sites of mutations associated with familial hypertrophic cardiomyopathy. Phosphorylation of the RLC can modulate cardiac contractility independently of troponin/tropomyosin-mediated regulation, but the molecular mechanism of such modulation is poorly understood. We are investigating structure-function relationships in the cardiac myosin RLC in the native environment of cardiac muscle cells. Pairs of cysteine residues were genetically introduced at six different pairs of surface-accessible sites in the RLC, each of which are 10-15 Å apart. Three pairs of cysteines were introduced in the N-terminal lobe of the RLC, and three in the C-terminal lobe. Each pair of cysteines was cross-linked with a bifunctional rhodamine: bis-[N-iodoacetyl(piperazyl)]-sulforhodamine (BSR; B-10621, Invitrogen) and the labelled protein purified to 95% homogeneity. The pure 1:1 BSR-RLC conjugates are exchanged into demembranated cardiac muscle cells and the orientation of the BSR fluorescence dipole determined by polarized fluorescence. With three probes on each lobe of the RLC, the orientation of the lobe with respect to the actin filament axis and the bend between the lobes can be determined. We are measuring these orientations under various physiological conditions including relaxation, active contraction, and rigor, and investigating the effects of RLC phosphorylation by myosin light chain kinase. These experiments should provide insight into RLC function in cardiac muscle and the mechanism of modulation of cardiac contractility by RLC phosphorylation.

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**702-Pos Board B502**

**EPR Spectroscopy Shows Oriented Myosin Heads in Relaxed Muscle Fibers**

Nariman Naber, Roger Cooke, **Edward Pate**.

The super relaxed state (SRX) is a newly discovered state of myosin in relaxed skinned skeletal fibers characterized by an ATP turnover-rate that is an order of magnitude lower than the turnover-rate measured for skinned fibers. X-ray diffraction and cryoEM show a high degree of order in the myosin heads in relaxed filaments. Conditions that perturb the ordered array also perturb the fraction of myosin in the SRX, suggesting the two are related and motivating the use of EPR spectroscopy to study the structure of the SRX. However, EPR has previously reported that the myosin heads are highly disordered in relaxed fibers. The sole exception was Baker and Thomas, who observed orientation of a probe on the RLC of myosin in scallop fibers. Using spin-labeled nucleotide-analog EPR spin probes and blebbestatin to stabilize the SRX in skeletal muscle, we now observe an oriented spectrum in relaxed fibers. A similar long-lived state is seen using epifluorescence of mant-nucleotides in skinned fibers in the presence of belbbestatin. We have repeated the experiments using skinned tarantula muscle. The filaments are dual regulated. Nucleotide-analog EPR spin probes report oriented myosin heads in relaxed fibers when the heads are dephosphorylated. Orientation disappears when the myosin heads are phosphorylated. EM likewise shows an oriented array of thick-filament myosin heads in dephosphorylated fibers that disappears in phosphorylated fibers (Craig, Padron, et al). Together, these data support the hypothesis that the SRX cross-bridges and the oriented array of cross-bridges seen in EM are the same.

**703-Pos Board B503**

**High-Resolution EPR of a Bifunctional Spin Label Reveals an ADP Induced Structural Rearrangement of the Actin-Bound Myosin Catalytic Domain**

**Ryan N. Mello**, Roman V. Agafonov, Andrew R. Thompson, David D. Thomas.

We have used electron paramagnetic resonance (EPR) of a bifunctional spin label (BSL) to measure structural transitions of the catalytic domain (CD) in *Dictyostelium* myosin II. The use of BSL is a critical feature in this work. The bifunctional attachment of BSL eliminates most of the nanosecond motions characteristic of monofunctional labels, making it possible to measure protein structural transitions with a precision not previously achievable. Two double-Cys constructs were engineered with Cys residues at helical locations i and i+4 (494.498 and 639.643). Residues 494.498 are located on the relay helix and residues 639.643 are located on a helix adjacent to the relay helix. After BSL labeling, myosin was bound to actin in oriented muscle fibers, making it possible to measure helix orientation relative to the fiber axis. Spectra were acquired in APO and ADP states. Simulation of the 494.498.BSL spectra demonstrates that in APO and ADP states there are two highly ordered populations of the relay helix. APO and ADP spectra contain the same two spectral components, but the distribution of these two components differs between states. Similarly, spectra from the 639.643 construct reveal an ADP-induced structural transition, but the difference between APO and ADP spectra demonstrates a 3° rotation of the 639.643 helix. Our results demonstrate structural transitions of two helixes within the CD of actin-bound myosin associated with nucleotide binding. Measuring these transitions is essential to understanding the molecular mechanism of force generation. This is particularly true for the relay helix, which plays a key role in the coupling of myosin ATPase and motor function. Additionally, these results demonstrate the utility of BSL for measuring transitions in protein orientation, order, and dynamics.

**704-Pos Board B504**

**Functional Mutations in the Force Generation Region Destabilize the Relay Helix in Myosin**

Roman V. Agafonov, Sarah Blakely, Margaret A. Titus, David D. Thomas, **Yuri E. Nesmelov**.

We have used pulsed EPR spectroscopy (DEER) to determine the structural effects of point mutations in the force generation region of myosin (I499A, F506A, F692A, *D.discoideum* sequence). As it was previously reported, these myosin mutants maintain basal ATPase activity, but completely lose their motor function. It was proposed that F506A, located in the relay loop, disrupts communication between the nucleotide binding site and the force generation region in myosin, and F692A and I499A, located on the interface with the converter domain, affect coordination of the converter domain relative to the myosin head. In this study we assayed the structure of the relay helix in these mutants and in wild-type myosin. The mutations were introduced into the A639C:K498C myosin construct, both Cys were labeled with maleimide spin probes, and interprobe distance was measured. Previous studies showed that probes at these sites detect the nucleotide-induced bending of the relay helix. Observed spin echo decays were interpreted in terms of one or two Gaussian distance distributions, corresponding to one or two myosin structural states. Nucleotide analogs were used to trap myosin in several biochemical states. In the wild type, two structural states, corresponding to pre- and post-recovery stroke, are present in a single biochemical state. The mutations affect the equilibrium between these two structural states and increase the conformational disorder, indicating that these mutants destabilize the structure of the force-generation region, particularly in the post-recovery state. These results provide a structural explanation for the functional perturbation, introduced to myosin.

**705-Pos Board B505**

**A Novel Actin Binding Site of Myosin is Responsible for Effective Muscle Contraction**

**Boglarka Varkuti**, Zhenhui Yang, Balint Kintses, Peter Erdelyi, Tibor Vellai, Miklos Kellermayer, Andras Malnasi-Csizmadia.

F-actin is a track protein for myosin motors as well as an activator of the myosin ATPase activity. Actin activation provides effective contraction by increasing the ratio of productive myosin heads over futile heads by several orders of magnitudes. Despite the functional significance of actin activation, its structural