EPR Analysis of Myosin Structural Dynamics

Robert Harris, Ryan Mello, Amanda Laden, David D. Thomas

Abstract

The structural dynamics of myosin during muscle contraction can be discerned in situ through site directed spin labeling of myosin and electron paramagnetic resonance (EPR) spectroscopy. To achieve in situ measurements, spin labeled myosin regulatory light chain (RLC) is exchanged for endogenous RLC in rabbit psoas fiber bundles. In order to ensure the structural integrity of exchanged muscle fibers, functional measurements must be done before and after exchange. After verifying function after RLC exchange, we can use EPR to measure the orientational dynamics of the RLC in a variety of states during muscle contraction.

Background

Muscle fibers are composed of myofibrils, which in turn are composed of sarcomeres. Each sarcomere contains actin and myosin filaments which slide past each other as a muscle contracts. The myosin head consists of two domains, the catalytic domain and the light-chain domain. The catalytic domain contains the ATP binding pocket, where ATP binds, and the actin binding cleft, which binds to actin as the muscle contracts.

The light chain domain consists of a single α-helix, extending from the catalytic domain, to which two light chains are bound, known as the essential light chain and the regulatory light chain (RLC). When myosin binds ATP, myosin is weakly bound to actin. Myosin then hydrolyzes ATP into ADP and P_i, using the chemical potential energy stored in ATP to do mechanical work. As ATP is hydrolyzed and P_i is released, the interaction between myosin and actin isomerizes from weak to strong, and force is generated (Figure 2). Afterwards, the muscle is again in a strongly bound state, ready to begin the process again. The weak and strong binding states have been studied extensively, however the states intermediate between weak and strong binding have not been studied as thoroughly. Previous work on the catalytic domain provides evidence that crosslinking SH1 and SH2 traps myosin in a pre-power stroke state. Using EPR spectroscopy, we can study the structural dynamics of the light chain domain in this pre-power stroke state.

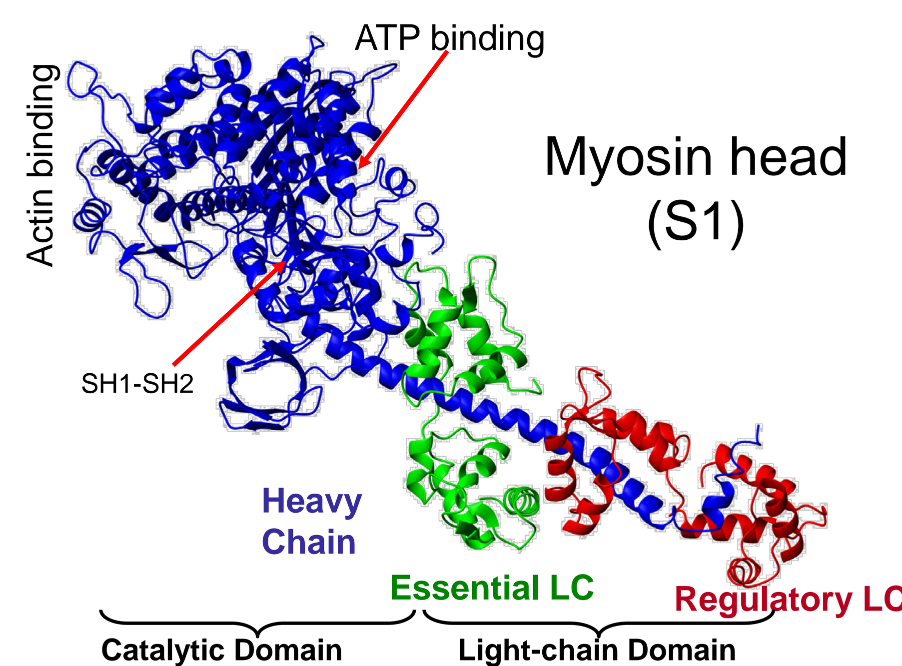


Figure 1: Myosin crystal structure

A.M.D ↔ A.M.T ↔ A.M.D.P ↔ A.M'.D.P ↔ A.M.D

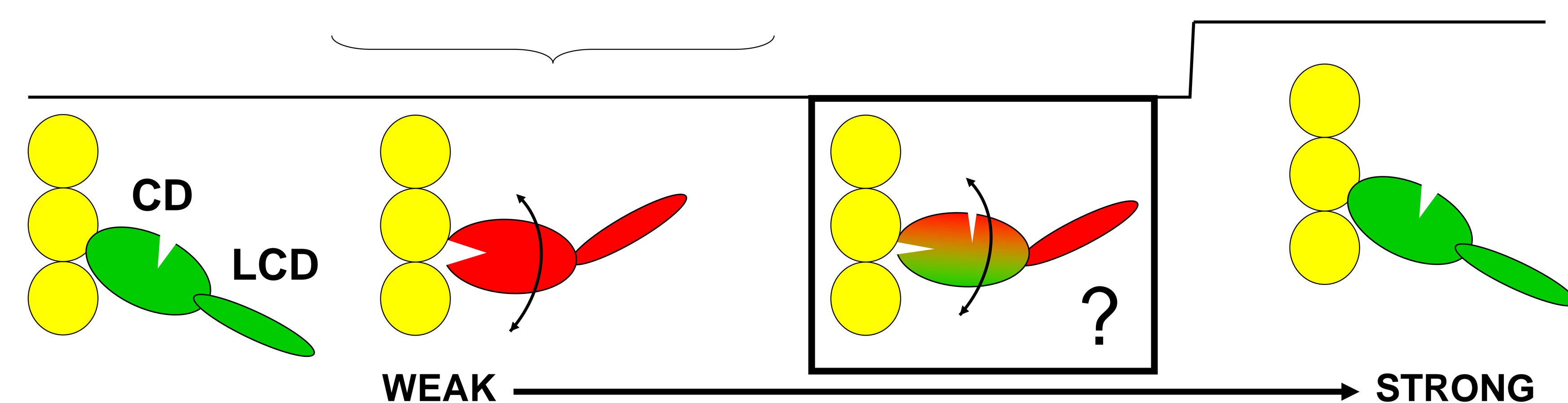


Figure 2: The interaction of myosin and actin upon muscle contraction. A pre-power stroke state is proposed to exist between the strongly and weakly bound states.

Methods

Exchange - In order to attach a spin label to the myosin RLC in muscle fibers, rabbit psoas fiber bundles are washed in pre-extraction solution followed by extraction solution. The extraction solution both removes the endogenous RLC and simultaneously reconstitutes the fiber with RLC that has been spin-labeled with FDNASL. In the case of our cross-linked fibers, the fiber bundles were also washed in pPDM following the RLC exchange.

Gel Electrophoresis - In order to verify we are sampling a significant portion of the myosin heads, we must determine how much endogenous RLC is being replaced with spin-labeled RLC. Control and exchanged fibers are homogenized and analyzed using gel electrophoresis. In this process, the homogenized fibers are placed in a polyacrylamide gel, giving them a negative charge. By applying an electric field across the gel, the negatively charged proteins will migrate towards the anode, separating the proteins by molecular weight. The spin label adds weight to the RLC; therefore, our spin-labeled RLC will form a band discernable from our endogenous RLC. By measuring the intensity of these bands, we can determine the amount of each protein in our fiber.

ATPase Assay - The calcium dependence of homogenized control and exchange fibers can be determined by an absorbance-based NADH-coupled ATPase assay. The rate of ATP hydrolysis in the presence of calcium is measured, which is representative of the functional integrity of the fiber.

EPR Analysis - From the EPR spectra of spin-labeled muscle fibers, the orientation of the RLC can be determined. By comparing the spectra from fibers in strongly bound, weakly bound, and cross-linked states we can determine whether the cross-linking has trapped our fibers in an intermediate state.

Results

Gel Electrophoresis - Our gel did indeed yield an additional protein band for the exchanged fibers, corresponding to the spin-labeled RLC. A plot of the optical density of the bands of relevance is given below. It indicates that we are removing about 30% of the endogenous RLC and replacing it almost entirely with spin-labeled RLC.

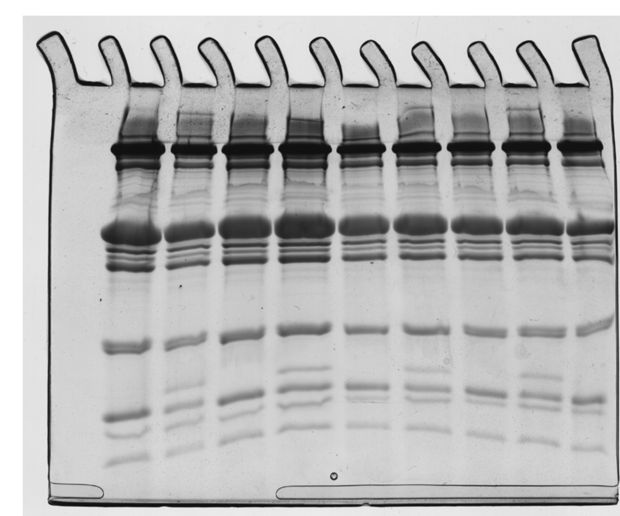


Figure 3: The results of running a gel on our homogenized fibers. Lanes 2, 4, 6, 8, and 10 are control fibers; lanes 3, 5, 7, and 9 are exchanged fibers.

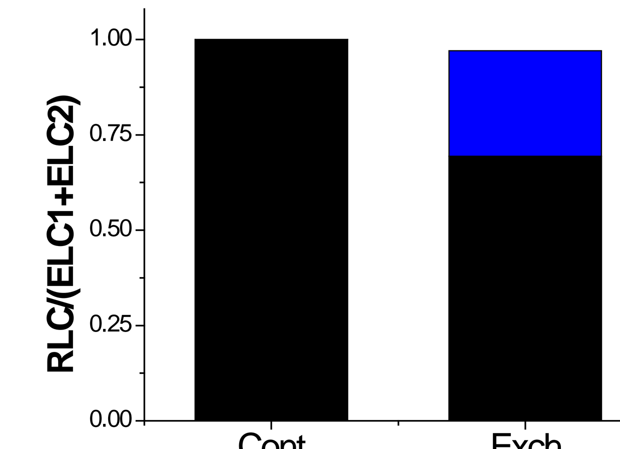


Figure 4: An optical density plot for the bands of interest in our gel. Black corresponds to endogenous RLC, and blue to the spin-labeled RLC.

ATPase Assay - The rate of ATP hydrolysis in homogenized control and exchanged fibers was shown to be very similar, indicating that the fibers retain their calcium dependence of ATP hydrolysis after RLC exchange.

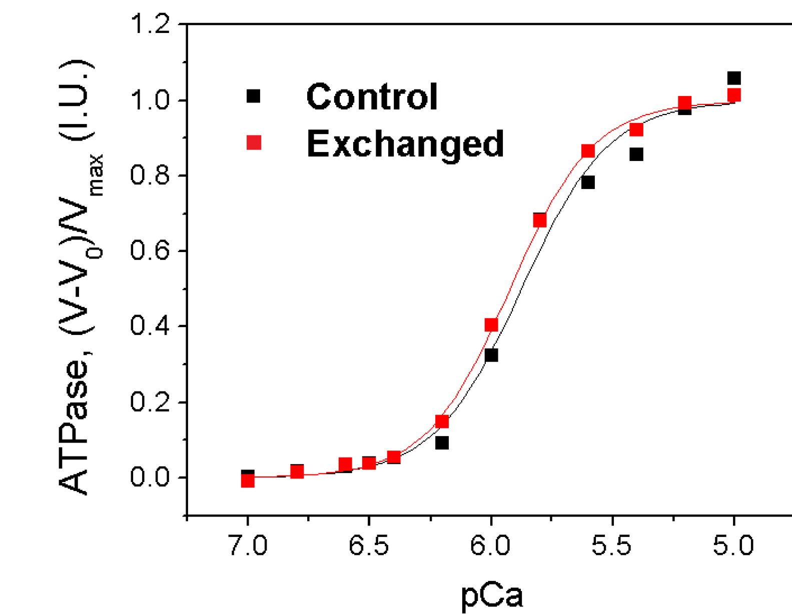
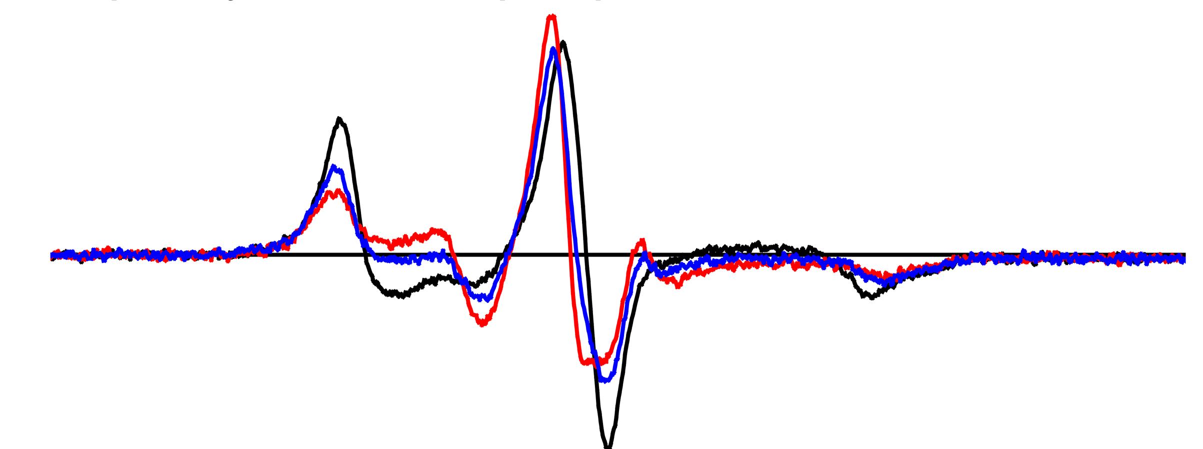


Figure 5: Results of ATPase assay on control and exchanged fibers.

EPR Analysis - The EPR spectrum of fibers containing the cross-linked catalytic domain is intermediate between the weakly bound and strongly bound spectra. This is consistent with the hypothesis that crosslinking SH1 and SH2 traps myosin in an pre-power stroke state.



(1)Before pPDM treatment (strongly bound)
(2) Before pPDM treatment (weakly bound)
(3) After pPDM treatment

Figure 6: The EPR spectra obtained from fibers in the three states of interest.

Conclusions

These results are consistent with the hypothesis of a pre-power stroke state existing between the weakly and strongly bound states when SH1 and SH2 are cross-linked. The RLC exchange process allows us to sample a significant portion of the myosin heads without damaging the function of the fiber. The EPR spectrum of the intermediate state lies intermediate between the spectra from the weakly and strongly bound states. These results suggest that cross-linking of SH1 and SH2 in the myosin catalytic domain using pPDM traps a state in which the myosin head is on the cusp of force generation, explaining myosin's structural transition from weak to strong binding as muscle contracts.

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Minnesota Muscle Laboratory