

Conformational changes of myosin leading to powerstroke

Theses of Ph.D. dissertation

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Introduction

Myosins are ubiquitous motor proteins in eukaryote cells, which generate the movement of molecules, organelles, cells, and whole organisms by powering translocation along actin filaments. Myosins have many forms performing different functions, however, they all follow the basic mechanochemical scheme shown in **Figure 1**. The basis of myosin's motor activity is the powerstroke step of this cycle. Upon this process, the release of ATP-hydrolysis products occurs, myosin forms strong interactions with the actin filament, myosin's lever swings and the distal part of myosin translocates relative to the actin filament.

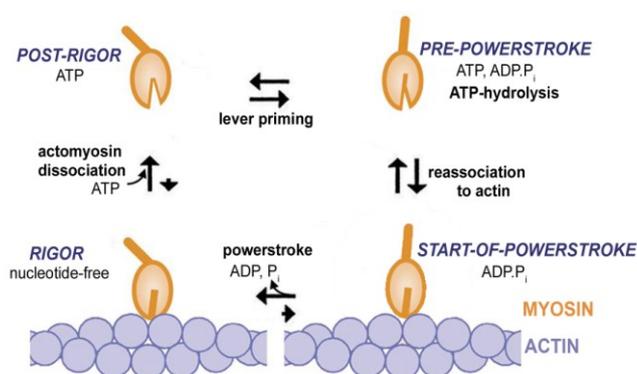


Figure 1: The mechanochemical cycle of actomyosin and nomenclature of the most important structures.

In spite of the long-standing efforts in myosin research the precise details of the powerstroke are still unresolved. The start point of the powerstroke is unknown, the properties of this myosin structure were only surmised. Furthermore, the end state of the powerstroke is the nucleotide-free rigor actomyosin complex, whose atomic structure is still lacking. Only actin-free rigor-like structures were described. Different myosin isoforms show distinct properties in their rigor-like crystal structure: the so-called actin binding cleft adopts different extent of closure. Considering that the cleft must close to form the rigor complex these differences of the rigor-like structures suggest diverse kinetic and energetic actin binding pathways for the isoforms (**Figure 2**).

In this work I summarize our new findings on the powerstroke step in the above aspects.

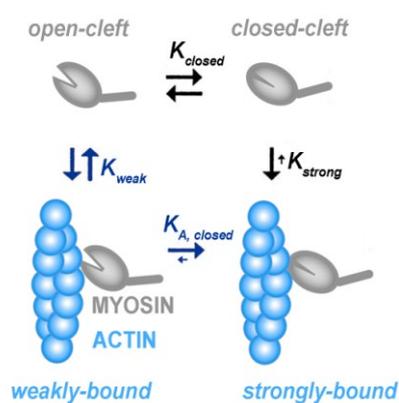


Figure 2: Structural and kinetic scheme of actin binding pathways. In the absence of actin, the myosin head (grey) may adopt open- and closed-cleft structures whose interconversion is dictated by the K_{closed} equilibrium constant (upper row). Myosins having an open cleft may follow the pathway shown by blue arrows (K_{weak} and $K_{A, \text{closed}}$) upon actin binding. Myosins crystallized with a closed cleft may bind to actin on the pathway depicted by black arrows (K_{strong}).

Objectives and questions

Our aim was to identify the structural changes of myosin occurring during the formation of the strong-binding actomyosin complex and the powerstroke. We applied the catalytic fragments (subfragment-1 – S1 – and motor domain) of different muscle and non-muscle isoforms of myosin 2, and that of the vesicle transporter myosin 5.

Problem 1: The start point of the powerstroke step is unknown.

Approach: We investigated the mechanism of blebbistatin, a myosin 2 inhibitor. My supervisor and his colleagues showed that blebbistatin binds to the bottom of the actin binding cleft and blocks the ATPase cycle in the pre-powerstroke state (in the M.ADP.P_i komplex).

Questions:

How does blebbistatin influence the conformational changes of myosin 2 motor domain?

Does blebbistatin change the coupling between the nucleotide binding pocket and the actin binding cleft and between the nucleotide binding pocket and the lever, respectively?

Through which conformational changes does the formation of the strong actin binding complex and the swing of the lever occur during the powerstroke?

Can we produce another stable conformational intermediate of the powerstroke besides the former identified myosin.ADP.P_i.blebbistatin complex?

Problem 2: Different myosin isoforms show distinct properties in their rigor-like crystal structures: the actin binding cleft exhibits different extents of closure. These differences of the rigor-like structures suggest diverse kinetic and energetic actin binding pathways for these isoforms.

Approach: We characterized and compared actin binding and actomyosin dissociation processes of myosin isoforms with different rigor-like structures.

Questions:

How does the energetic and kinetic profile of the formation of the strong actomyosin interaction relate to the structure of different myosin isoforms in the absence of actin?

How do conformational changes occurring upon strong actin binding (e.g. cleft closure) influence the energetics of actin binding process?

To what extent do the energetic changes contribute to the powerstroke step?

How universal are the energetic changes upon powerstroke among the different myosin isoforms?

Experimental investigation

Applied methods

Protein expression in eukaryotic cultures: *Dictyostelium discoideum* (wild-type and single-tryptophan myosin 2 motor domains – DdMD) and Sf9-baculovirus (myosin 5 S1 – m5S1) systems.

Protein purification: His- (DdMD) and FLAG-tag (m5S1) affinity chromatography.

Protein preparation from rabbit skeletal muscle: myosin 2 S1 and actin.

Chemical modification of actin: labeling on Cys³⁷⁴ with N-(1-pyrene)iodoacetamide.

Photometric ATPase activity measurement: NADH-coupled assay.

Steady-state and equilibrium fluorimetry.

Transient kinetics: fluorescent stopped-flow experiments.

Actomyosin cosedimentation assay, SDS-PAGE of the fractions and densitometry of the gels.

Isothermal titration calorimetry (ITC).

With the support of collaborators: electron-microscopy and atomic-level calculations.

Experiments answering the questions

We searched the effect of blebbistatin on the conformational changes of myosin using wild-type and single-tryptophan mutant DdMD constructs. Fluorescence changes of the mutants have been assigned to the conformational changes of myosin: W501+ mutant – which contains its single tryptophan at the base of the lever – is a lever sensor, W239+ myosin – which contains its tryptophan in the switch-1 loop at the active site – indicates cleft closure *via* allosteric communication. We measured fluorescence changes and the kinetics of the changes of these sensors in the presence and in the absence of ADP, ATP, ADP.AIF₄⁻ (ADP.P_i-analogue) and blebbistatin, respectively. We applied wild-type DdMD myosin and pyrene-labeled actin to investigate the strong-binding actomyosin interaction. In collaboration, we investigated the corresponding myosin structures by electron microscopy.

We studied the conformational and energetic changes leading to the formation of the rigor actomyosin complex comparing four different myosin isoforms whose rigor-like structures are known. We examined actomyosin binding and dissociation processes using pyrene-actin in different independent ways. We calculated energetic parameters from the temperature dependence of these processes. We measured the energetics of actomyosin binding directly by calorimetry. In collaboration, we performed energetic calculations based on the atomic structures of the myosin isoforms.

Results (theses)

Result 1: Blebbistatin inhibits ATPase activity of all studied DdMD constructs.

Result 2: Blebbistatin induces lever priming in the myosin.ADP complex.

Result 3: Blebbistatin does not change the actin binding properties of myosin.

Result 4: Myosin motor domains populate a strongly actin-bound state with a primed lever in the actomyosin.ADP.blebbistatin ternary complex.

Result 5: Blebbistatin binding does not change the structure of apo myosin.

Result 6: The investigated isoforms showed similar fluorescence quench upon binding to pyrene-actin.

Result 7: The energetic profile of actin binding corresponds the cleft closure propensities observed in rigor-like crystal structures. Myosins crystallized with closed cleft bind to actin exothermically.

Result 8: The actin-binding rate constant and affinity of different myosins does not directly correlate with the energetic character of actin binding and cleft conformation.

Result 9: Upon cleft closure, the torsion of myosin's central β -sheet 'transducer' appears very similar in all isoforms according to our atomic-level calculations.

Result 10: Actin binding by myosin heads is markedly more endothermic in the ADP-bound state than in the nucleotide-free form in all investigated isoforms, and the difference is similar in all cases.

Conclusions

Conclusion 1: Blebbistatin is an effective inhibitor of the ATPase activity of our DdMD constructs, and thus these constructs are good models to study the mechanism of blebbistatin.

Conclusion 2: According to the fluorescence changes of lever sensor W501+ mutant, in the presence of blebbistatin the lever shows priming not only in ATP, but also in ADP (i.e. in the absence of a ligand at the γ -phosphate pocket); myosin thus adopts the pre-powerstroke conformation. Our collaborators confirmed the primed-lever state in the myosin.ADP.blebbistatin complex by electron microscopy. This is the first primed-lever myosin structure without any ligands at γ -phosphate pocket. In agreement this finding we showed that blebbistatin inhibits filling of γ -phosphate binding site, which indicates that myosin.ADP.blebbistatin complex has an inaccessible γ -phosphate pocket.

Conclusion 3: According to the fluorescence changes of the cleft sensor W239+ mutant, myosin retains the equilibrium of actin binding: high actin affinity in ADP and low actin affinity in ATP in the presence of blebbistatin as well. We justified the fluorescent results with actomyosin cosedimentation experiments.

Conclusion 4: Tryptophan fluorescence changes of acto-W501+ showed the same tendency as in the absence of actin. This result proves that in blebbistatin the presence of actin does not influence the distribution of lever states: ADP binding of acto-W501+ also induces lever priming. We found strong actin binding of myosin.ADP.blebbistatin complex in the case of all DdMD constructs: detecting pyrene-actin and tryptophan fluorescence, as well as light scattering in steady-state and transient kinetic experiments, and also by cosedimentation. The primed-lever, high actin-affinity myosin intermediate we captured in the presence of blebbistatin and ADP has the same properties as the surmised start point of myosin's powerstroke.

Conclusion 5: The active and inactive enantiomers of blebbistatin (the latter does not bind to W501+ myosin at all) cause similar tryptophan fluorescence quench in the case of the two DdMD mutants. Thus, this decrease is not due to the intrinsic tryptophan fluorescence change of myosin, but to inner filter effect (originating from light absorption) of blebbistatin. It means that blebbistatin binding alone (in the absence of actin and nucleotides) does not induce structural changes in the apo motor domain.

Conclusion 6: Despite the different extents of cleft closure in the rigor-like structures, the actomyosin interface is similar in all myosin isoforms.

Conclusion 7: We derived energetic data from temperature dependence of kinetics of actin binding and actomyosin dissociation. We confirmed the obtained values by fluorescent equilibrium titrations and by calorimetric experiments of actin binding. Open-cleft rabbit muscle myosin 2 binds to actin endothermically, in line with former reports. In contrast, we observed exothermic actin binding in the case of closed-cleft molluscan muscle myosin 2, DdMD and myosin 5.

Conclusion 8: Contrary to previously proposed ideas, we found that the cleft closure propensity in the absence of actin (K_{closed} , **Fig. 2**) does not directly dictate either the magnitude of the second-order actin binding rate constant or the actin binding affinity. Although K_{closed} could not be determined directly, available structural data and our energetic calculations indicate that the equilibrium is pulled either to the closed-cleft state (exothermic actin binding) or to the open-cleft state (endothermic actin binding). As the cleft closure propensity and actin affinity of the isoforms do not correlate, the different myosins show diversity not only in

K_{closed} equilibrium constants, but also in the actin affinities in weakly- or strongly-bound states (K_{weak} and K_{strong} , **Fig. 2**).

Conclusion 9: The transducer β -sheet located in the core of the motor domain may play an important role in the allosteric communication between the actin and nucleotide binding sites *via* changes in its torsional strain. Our calculations strengthen the idea that this torsion is necessary for the powerstroke. However, it cannot explain the energetic differences observed at different isoforms. Based on our results, we propose that the transducer is mechanically more strained in actin-bound myosin: an internal strain evolves within myosin heads upon cleft closure in order to adopt precise steric complementarity to the actin filament.

Conclusion 10: We studied the energetic character of actomyosin interaction in the presence of ADP. The nucleotide induced the open-cleft (post-rigor) myosin conformation. Enthalpic changes defined in the direction of cleft closure (post-rigor to rigor-like/rigor) suggest that cleft closure is an enthalpically unfavorable process. However, the large favorable electrostatic contribution arising from interactions during cleft closure surmount this enthalpic cost.

Summary

Myosin populate a primed-lever, high actin-affinity state in ternary complex with ADP and blebbistatin inhibitor. Primed-lever myosin was known only in the presence of ATP or some other ligands occupying the γ -phosphate binding site. The characterized intermediate is likely the start point of the powerstroke step. According to this finding the formation of the strong actin binding complex induces the powerstroke step. Upon formation of the strong-binding actomyosin complex, the actin binding cleft of myosin must close. This is an endothermic process in vertebrate skeletal muscle myosin. In contrast, molluscan and DdMD myosin 2, as well as myosin 5 which have a closed cleft even in the absence of actin bind to actin exothermically. In this way, cleft closure propensity determine the energetics of actin binding and, consequently, the powerstroke step.

Publications

Publications concerning this thesis:

Balázs Takács, Neil Billington, Máté Gyimesi, Bálint Kintses, András Málnási-Csizmadia, Peter J. Knight, and Mihály Kovács (2010): Myosin complexed with ADP and blebbistatin reversibly adopts a conformation resembling the start point of the working stroke. *Proc Natl Acad Sci U S A*, **107**, 6799-6804.

Balázs Takács, Elizabeth O’Neill-Hennessey, Csaba Hetényi, József Kardos, Andrew G. Szent-Györgyi, and Mihály Kovács (2010): Myosin cleft closure determines the energetics of the actomyosin interaction. *FASEB J*, [Epub ahead of print].

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