Structural Background of Force Generation in Myosins

Theses by Boglárka Várkuti

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Introduction

P-loop NTPase activation by allosteric effectors commonly occurs in various motor and signaling systems, such as actin-myosin, microtubule-kinesin, GEF-G-protein or DNA-DNA polymerase complexes. Although its significance in eukaryotic cell functioning, the structural mechanism of activation is still unrevealed.

F-actin serves as a track for myosin's motor functions and activates its ATPase activity, enabling actomyosin to produce effective force against load. The rate limiting step of the myosin II enzyme cycle in the absence of actin is the up-to-down lever swing i.e. the reverse recovery step, determining the basal steady-state ATPase activity of myosin. Actin accelerates the reverse recovery step, thereby enabling other steps of the myosin enzyme cycle to become rate-limiting in the presence of actin. As a result, myosin's steady-state ATPase activity increases by several orders of magnitude.

Actin activation is carried out by actin binding weakly to myosin in its pre-powerstroke state, creating the initial powerstroke state of the myosin enzyme cycle. During powerstroke, which is the actin-attached up-to-down lever swing, actin-binding strengthens and phosphate is released from the actomyosin complex. Actin activation channels myosin heads into the effective route of the myosin enzyme cycle by the kinetic selection pathway mechanism, leading through the powerstroke step. Although actin activation is a common mechanism among myosins, its structural background has been unrevealed, mainly due to the limited knowledge of the structures of the initial, end and intermediate states of the powerstroke. Even the structural element of myosin specifically responsible for actin activation is unrevealed.

Recently, a possible start-of-powerstroke, or intermediate powerstroke state of myosin has been detected in the presence of ADP and blebbistatin, which is a myosin II specific inhibitor. The crystallization of the myosin-ADP-blebbistatin complex has been hindered due to the low affinity of blebbistatin to the myosin-ADP complex. Therefore, the atomic structure of an important state of the powerstroke remains yet unrevealed.

Aims

In order to reveal insights into the mechanism of actin activation of myosin, the following aims are addressed:

- reveal the myosin structural element specifically responsible for the actin activation of myosin
- examine the specific role of actin activation of myosin *in vivo*
- determine the atomic structure of the initial, weak-binding actomyosin state of the powerstroke
- discover communication pathways from the actin-binding region to the nucleotide binding pocket and converter/lever region of myosin
- enhance the crystallization of the myosin-ADP-blebbistatin complex by covalent cross-linking of blebbistatin to myosin in the presence of ADP

Theses - Results

1. *We described a novel interaction between a conserved loop of myosin and the N-terminal region of actin, which interaction is specifically responsible for the actin activation of myosin. We named this newly discovered actin-binding loop of myosin as activation loop.*

We conducted *in silico* docking of an actin trimer model and weak actin-binding up lever (PDB code: 1VOM), down lever (1MMD) and strong actin-binding (2OVK) myosin structures. Molecular dynamic relaxation of the actomyosin complexes and *in vitro* cross-linking experiments revealed a novel actin-binding loop that creates salt bridge interactions with the N-terminal region of actin. We characterized the steady-state and transient kinetic properties of the actin-binding loop mutant *Dictyostelium discoideum* myosin II motor domain (*Dd* MD) constructs, which were similar to the wild type in the absence of actin. Mutations in the loop slightly affected the actin-binding of *Dd* MD, whereas they almost completely abolished the actin activated ATPase activity of myosin, therefore we named the newly discovered actin-binding myosin region as activation loop. The decrease of actin activation was also observed in activation loop mutant *Mus musculus* myosin Va S1 (myoV).

2. *Actin activation and* in vitro *motility of myosin is decoupled upon abolishing the interaction between activation loop of myosin and the N-terminal region of actin.*

Activation loop mutant *Dd* MD constructs incapable of being activated by actin are able to move actin filaments with similar velocities to the wild type *Dd* MD in *in vitro* motility assays.

3. *Actin activation of myosin's ATPase activity enables* in vivo *force-generation of the actomyosin system.*

We created transgenic *Caenorhabditis elegans* (*C. elegans*) strains, containing wild type or activation loop mutant body-wall myosin. Animals bearing a mutation in their activation loop of body-wall myosin moved faster than the body-wall myosin knockout worms, although slower than the wild type animals. In contrast, force-generation of activation loop mutant worms was completely abolished compared to wild type animals, and were similar to body-wall myosin knock out *C. elegans*.

4. By in silico *methods we determined the atomic structure of the weak-binding actomyosin complex representing the initial state of the powerstroke, where myosin is in an 'extra primed', novel structural state.*

We carried out *in silico* docking of an actin trimer and a myosin motor domain in its up-lever, weak actin-binding state (1VOM) and performed a 100 ns-long molecular dynamic relaxation of the resulted weak-binding actomyosin complex. By the same method, we determined structures of the strong-binding rigor actomyosin (actin trimer + apo/rigor-like crystal structures, 1Q5G/2OVK), and compared the results to existing rigor complexes for the validation of our method. The weak-binding actomyosin complex relaxed to an 18^{o} further up lever accompanied with a further closed switch II structural state of myosin upon molecular dynamics, which represents a novel, 'extra primed', initial state of the powerstroke.

5. *The interaction between activation loop of myosin and actin N-terminal region is responsible for the extra primed state of myosin in the initial powerstroke state.*

In silico mutations that abolish the interaction between activation loop and actin N-terminal region cause the 'back-relaxation' of the extra primed state of myosin upon molecular dynamics. *In silico* mutations in other actin-binding loops of myosin cause no such effect.

6. We revealed a weak actin-binding induced structural communication pathway originating from the actin-binding activation loop towards the relay region and nucleotide-binding pocket of myosin.

Upon the formation of the interaction between the N-terminal region of actin and activation loop of myosin in the weak actomyosin complex, the upper relay region (where activation loop is situated) becomes interconnected with the relay helix and switch II loop of the nucleotide-binding pocket through salt bridge and hydrophobic interactions, causing the formation of the extra primed state of myosin. *In silico* mutation of activation loop abolishes this structural communication pathway between

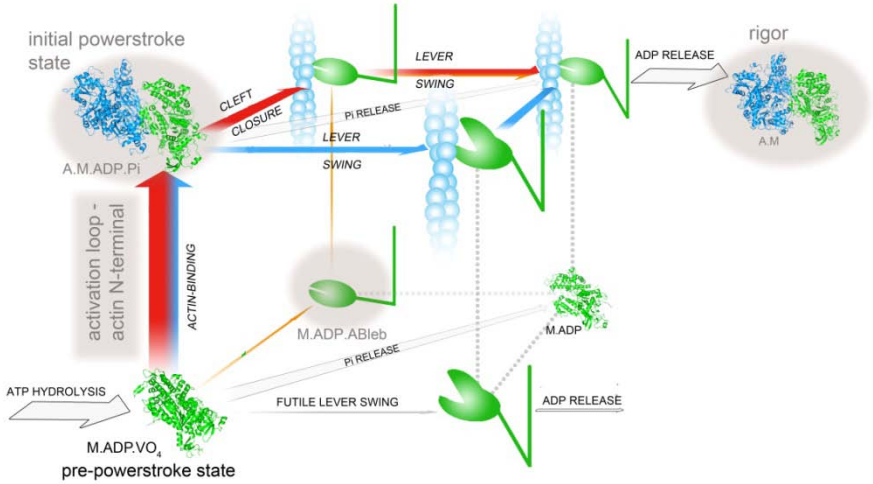
the actin-binding region, lever and nucleotide-binding pocket of myosin, while other actin-binding loop mutations leave the interactions intact.

7. *We covalently saturated myosin with a photoreactive derivative of blebbistatin, para-azidoblebbistatin in the presence of ADP, thereby enabling the crystallization of the myosin-ADP-azidoblebbistatin complex.*

We synthesized para-azidoblebbistatin, the C15 azido-derivative of blebbistatin, which can be covalently cross-linked to myosin upon photo-activation by UV irradiation. Para-azidoblebbistatin inhibits *Dd* MD ATPase activity similarly to that of blebbistatin in the absence of UV irradiation. By a series of cross-linking cycles, we were able to saturate myosin in the presence of ADP by para-azidoblebbistatin. We also identified the myosin binding site of azidoblebbistatin in the presence of ADP.

Conclusions

- elimination of the salt bridge interactions between activation loop and actin N-terminal region abolishes the activation of myosin's ATPase activity, leaving other parameters of the enzymatic cycle intact, which demonstrates the specific role of activation loop in the actin activation of myosin
- force-generation of muscles *in vivo* is regulated by actin activation of myosin through the interaction between activation loop and actin, tuning the efficiency of the actomyosin system
- the interaction between activation loop and actin induces the extra primed state of myosin in the initial powerstroke actomyosin complex, the atomic structure of which has never been resolved before
- the actin-binding region communicates with both the relay/converter structural domains and the nucleotide-binding pocket of myosin, which structural communication pathways are initiated by the formation of the interaction between activation loop and actin N-terminal region
- structural knowledge of an intermediate state of the powerstroke i.e. resolving the atomic structure of the M.ADP.ABlebb state would lead us further in the understanding of the molecular mechanism of force-generation in myosins



The mechanistic model of parallel powerstroke pathways of myosin. Known and unknown structures of different states of the mechanistic model are showed by cartoon or schematic representations of the myosin motor domain (green) and actin (blue), respectively. Structural states resolved in this study or contributions to reveal a structure (initial powerstroke state, rigor and M.ADP.ABleb) are highlighted by grey background, as well as activation loop–actin interaction revealed in this study, which enables the allosteric activation of myosin by actin. The widths of the arrows represent the relative fluxes of the pathways. The red, blue and orange pathways are three possible routes of the powerstroke. (PDB codes: 1VOM, pre-powerstroke; 1MMA, M.ADP)

Applied Techniques

- after the generation of activation loop mutations in *Dictyostelium discoideum* myosin II motor domain (*Dd* MD) by megaprimer PCR method and *Mus musculus* myosin Va S1 (myoV) by QuikChange mutagenezis, *Dd* MD constructs were expressed in *Dictyostelium* cell cultures while myoV constructs were produced in baculovirus–Sf9 (*Spodoptera frugiperda*) expression system
- protein purification was achieved by His- (Dd MD) and Flag-tagged (myoV) affinity chromatography
- actin preparation was carried out from rabbit skeletal muscle, chemical modification was applied by labeling Cys374 of actin with pyrene (N-(1-pyrene)iodoacetamide)
- zero-length covalent cross-linking of *Dd* MD and actin was achieved by EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide)
- transient kinetic fluorescence measurements of *Dd* MD were performed by BioLogic SFM 300 stopped-flow, following intrinsic Trp (W501), mant-dADP (3'-(N-methylantraniloyl)-2'-deoxy–ADP) and pyrene-actin fluorescence changes on a transient time scale

- steady-state basal and actin activated ATPase activities of *Dd* MD and myoV was measured by a pyruvate kinase/lactate dehydrogenase coupled assay in a Schimadzu UV-2101 PC spectrophotometer
- *Dd* MD–actin steady-state co-sedimentation assay was carried out by ultracentrifugation, SDS-PAGE and densitometry of the gels
- *in vitro* motility of TRITC-labeled (tetramethylrhodamine-5-isothiocyanate) actin filaments was visualized by Olympus IX71
- transgenic *Caenorhabditis elegans* (*C. elegans*) strains were produced by microparticle bombardment
- egg-production, lifespan and motility of *C. elegans* were quantified
- atomic force microscopy (AFM) using AsylumResearch MFP3D was applied to measure force-generation of *C. elegans*
- *in silico* preparation of protein structures was performed by Zhenhui Yang: VO_4^{3-} and BeF_3^- in 1VOM and 1MMD structures were replaced by Pi using Antechamber and Gaussian, missing residues of 1VOM, 2OVK and 1MMD were completed by homology modeling using Modeller 9.2, missing N-terminal region of actin was extended and capped with acetylate group (Acetyl-Asp_1-Glu-Asp-Glu_4-actin)
- actin-myosin docking was accomplished by Haddock V2.1 by Zhenhui Yang
- molecular dynamic relaxation was carried out by Zhenhui Yang using Amber
- chemical synthesis of para-azidoblebbistatin was performed by Miklós Képiró
- covalent cross-linking of *Dd* MD and para-azidoblebbistatin was achieved by photoactivation of myosin-bound para-azidoblebbistatin
- identification of para-azidoblebbistatin binding-peptides of myosin was achieved by mass spectrometry (by Miklós Képiró) and using Findmod program

Publications Concerning this Thesis

Boglárka H.Várkuti, , Zhenhui Yang, Bálint Kintses, Péter Erdélyi, Irén Bárdos-Nagy, Attila L. Kovács, Péter Hári, Miklós Kellermayer, Tibor Vellai and András Málnási-Csizmadia. "A novel actin binding site of myosin required for effective muscle contraction." Nature structural & molecular biology 19, no. 3 (2012): 299-306.

Miklós Képiró, **Boglárka H. Várkuti**, Andrea Bodor, György Hegyi, László Drahos, Mihály Kovács and András Málnási-Csizmadia. "Azidoblebbistatin, a photoreactive myosin inhibitor." Proceedings of the National Academy of Sciences 109, no. 24 (2012): 9402-9407.

Boglárka H. Várkuti, Zhenhui Yang and András Málnási-Csizmadia. " A structural model of actomyosin in the prepowerstroke state: actin induced 'extra priming' of myosin " – under submission

Conference Proceedings (the presenting author is underlined)

A structural model of actomyosin in the prepowerstroke state: actin-binding induced extra priming of myosin
Boglárka Várkuti *Zhen Hui Yang, András Málnási-Csizmadia*
2013 Philadelphia, US, Biophysical Society 57th Annual Meeting, poster presentation

An actomyosin model of the initial state of the powerstroke
Boglárka H. Várkuti*,Yang Zhe Hui, Málnási-Csizmadia András*
2012 Rhodes, European Muscle Conference, oral presentation

Structural Model of the Pre-Powerstroke State of the Actomyosin Complex
Zhen Hui Yang, ***Boglárka H. Varkuti****, Anna Rauscher, Miklos Kepiro, Andras Malnasi-Csizmadia*
2012 San Diego, US, Biophysical Society 56th Annual Meeting, oral presentation

A novel actin binding site enables effective muscle contraction
Boglárka H. Várkuti*, Bálint Kintses, Zhen Hui Yang, Péter Erdélyi, Irén Bárdos-Nagy, Péter Hári, Miklós Kellermayer, Tibor Vellai, András Málnási-Csizmadia*
2011 Berlin, European Muscle Conference, oral presentation

Synthesis and functional characterization of azido-blebbistatin, a photoreactive myosin inhibitor
Miklós Képiró, ***Boglárka H. Várkuti****, György Hegyi, Mihály Kovács, András Málnási-Csizmadia*
2011 Berlin, European Muscle Conference, oral presentation

Sythesis and functional characterization of azido-blebbbistatin, a photoreactive myosin inhibitor
Miklós Képiró, ***Boglárka Várkuti*** *and András Málnási-Csizmadia*
2011 Budapest, Hungary, 4th European Conference on Chemistry for Life Siences, poster presentation

A novel actin binding site of myosin is responsible for effective muscle contraction
Boglárka Várkuti*, Bálint Kintses, Zhen Hui Yang, Péter Erdélyi, Irén Bárdos-Nagy, Péter Hári, Miklós Kellermayer, Tibor Vellai, András Málnási-Csizmadia*
2011 Baltimore, US, Biophysical Society 55th Annual Meeting, poster presentation

The molecular mechanism of actin activation of myosin
Boglárka Várkuti*, Bálint Kintses, Zhen Hui Yang, András Málnási-Csizmadia*
2009 Lille, France, European Muscle Conference, poster presentation

The role of the proline-rich loop in the enzyme mechanism of myosin II
Boglárka Várkuti*, Kintses Bálint, Yang Zhenhui, Málnási Csizmadia András*
2008 Szeged, Annual Meeting of the Hungarian Society of Biochemistry, poster presentation, poster prize

The role of the proline-rich loop in the communication of actomyosin
Boglárka Várkuti*, Málnási-Csizmadia András*
2007 Temesvár, 10. International Students' Conference of Technological Sciences, oral presentation, special award

Other Publications

Miklós Képiró, **Boglárka H. Várkuti**, László Végner, Gergely Vörös, Máté Varga, András Málnási-Csizmadia. "Para-nitroblebbistatin, the non-phototoxic and photostable myosin II inhibitor." – under submission

Gene Technology and Protein Engineering, chapter 15 (electronic lectures, editor: Prof. László Nyitray) authors: Anita Alexa, Krisztián Fodor, Ágnes Garai, Gábor Glatz, László Radnai, Péter Rapali, Dávid Szakács, **Boglárka Várkuti**, András Zeke. Budapest 2013.