Structural Model of Weak Binding Actomyosin in the Prepowerstroke State

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Background: Actomyosin generates mechanical force in all eukaryotic cells including muscle.

Results: By dynamic computational simulations we revealed structural rearrangements in myosin upon actin binding, leading to the initial state of force generation.

Conclusion: The actin binding-induced structural rearrangements in myosin are transmitted specifically through the activation loop of the myosin.

Significance: The first actomyosin atomic structural model of the initial state of force generation.

We present the first in silico model of the weak binding actomyosin in the initial powerstroke state, representing the actin binding-induced major structural changes in myosin. First, we docked an actin trimer to prepowerstroke myosin then relaxed the complex by a 100-ns long unrestrained molecular dynamics. In the first few nanoseconds, actin binding induced an extra primed state of myosin specifically through the actin N terminus-activation loop interaction. The applied in silico methodology was validated by forming rigor structures that perfectly fitted into an experimentally determined EM map of the rigor actomyosin. Our results unveiled the role of actin in the powerstroke by presenting that actin moves the myosin lever to the extra primed state that leads to the effective lever swing.

Myosin is a ubiquitous molecular motor that generates force and moves along the actin filament. However, actin is not only a passive track for myosin but also its allosteric activator (1–3). The steady-state ATPase activity of myosin is accelerated by several orders of magnitude due to its cyclic interactions with actin. The mechanically effective step of the actomyosin cycle is the powerstroke (2, 4). Three enzymatic events must be harmonized to result in the powerstroke: actin re-binding (after the recovery step and ATP hydrolysis of myosin), the up-to-down lever swing, and actin-binding cleft closure of the myosin head leading to strong actin binding (Fig. 1). Actin re-binding to the up-lever M–ADP–Pi4 state is weak, thermodynamically unfavorable, however, mechanistically the resulting weak actomyosin complex represents a key enzymatic state, i.e. the primed pre-powerstroke state of the motor system. Actin must bind to the lever-up state of myosin to produce a mechanically effective, up-to-down lever swing, otherwise a non-productive, futile cycle occurs (4). Although actin binding to M–ADP–P_i is thermodynamically unfavorable, a kinetic pathway selection mechanism channels the cycle into the effective route by the actin-driven acceleration of the rate-limiting lever swing (4). In other words, in the absence of actin the rate-limiting step is the up-to-down lever swing (5), which is accelerated by actin, resulting in a large flux toward the effective lever swing pathway (Fig. 1). Recently, we found that the activation loop located in the upper part of the relay region of myosin binds to the N-terminal segment of actin (6). This actomyosin interaction specifically accelerates the rate of the up-to-down lever swing of M–ADP–P_i, thus it is responsible for maintaining the high ratio of flux of the effective versus futile lever swing pathways. Consequently, mutations that weaken the interaction between actin and the activation loop reduce the efficiency of the actomyosin system (6). Nevertheless, the molecular mechanism of the actin-induced acceleration of the lever swing is still unrevealed. This leads us to the fundamental question: what are the conformational rearrangements that occur upon actin binding to the up-lever, pre-powerstroke state of myosin?

The answer to this question is not easily accessible, as there are no crystal structures of the actomyosin complex. Atomic structural models of the rigor actomyosin complex have been presented based on high resolution electron microscopy (7–10). Recently, ATP-induced actin dissociation from myosin has been modeled by in silico simulations (11). To understand the mechanism of the powerstroke, there is a great demand to solve the initial state of the powerstroke, i.e. the weak A–M–ADP–Pi up-lever state complex. Determination of this

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4 The abbreviations used are: M, myosin; A, actin; AM, actomyosin; up, up-lever; dock, docked; rel, relaxed; ch, chicken (Gallus gallus) myosin; sq, squid (Doryteuthis pealei); PDB, Protein Data Bank.
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FIGURE 1. Structural changes in myosin during its chemomechanical cycle. The initial state of the powerstroke is the weak actomyosin ADP-Pi complex with an up position of the lever (prepowerstroke, highlighted with orange box). The magnitudes of the gray arrows refer to the flux of the reactions. The lengths of the black arrows represent the relative magnitudes of the rate constants of the steps of the enzyme cycle. The futile reaction pathway is represented with faded colors.

Weak binding actomyosin complex is experimentally challenging due to its low proportion and very short lifetime in equilibrium and steady-state systems, respectively. Thus, despite serious efforts (12), no high resolution EM-based structure is available of any weak actin-binding actomyosin complex. Therefore, we have determined the atomic model of the weak actomyosin complex with an up position of the lever (prepowerstroke, highlighted with orange box). The magnitudes of the gray arrows refer to the flux of the reactions. The lengths of the black arrows represent the relative magnitudes of the rate constants of the steps of the enzyme cycle. The futile reaction pathway is represented with faded colors.

EXPERIMENTAL PROCEDURES

Protein Structures Preparation—The Dictyostelium discoideum myosin 2 motor domain up-lever state (Protein Data Bank code 1VOM (PDB)) was extended with the missing amino acids (Ala205 to Ser208, Asn711, Ala716 to Ser719, Asp724 to Leu730, and Ala748 to Glu759) based on the structure by Jon Kull (referred to as Mapo) (13). Coordinates of all atoms of the Dictyostelium myosin 2 motor domain apo state (PDB 1Q5G, referred to as Mapo,Dd) are well defined. Vanadate in the nucleotide-binding pocket of PDB 1VOM was replaced by Pi. We calculated the partial charges and coordinates using the AMBER force field. The ADP parameters applied in the AMBER force field were provided by Meagher et al. (14). Restrained electrostatic potential charges of H2PO4− (the most dominant ionized state at pH 7.2, verified by quantum mechanical calculations under CHARMM force field) were calculated by Gaussian 03 and ANTECHAMBER (15, 16). The calculations were carried out for a set of structural conformations of the H2PO4− group by covering the accessible space in the system. The density functional algorithm with the B3LYP exchange and the 6–31+G (d,p) basis set were selected for calculation in Gaussian 03. The resulting charges were averaged over all of the conformations. All crystal waters were stripped except for two in the nucleotide binding pocket of PDB 1VOM, which stabilize the position of ADP and Pi, through interactions with the magnesium ion. The missing N terminus of the actin trimer (17) was sequentially extended and capped with acetylated (acetyl-Asp-Glu-Asp-Glu-actin) preceding the molecular minimization.

Protein-Protein Docking—After a 20-ns long molecular dynamic relaxation of the prepared crystal structures (Mapo,Dd, Mapo,Dd and Mapo,Dd), the averaged myosin structures were docked to the refined actin trimer using the HADDOCK program (High Ambiguity Driven Docking), resulting in (Mapo,Dd, dockMtr,Dd, and dockMtr) intermolecular restraint residues for docking were defined by the experimentally determined actomyosin interface (18–20), regarded as flexible segments and divided into active and passive amino acids for protein-protein docking. Active residues take part in actomyosin binding and passive residues are the neighboring residues of active residues. Overall, six active and 10 passive residues were selected from myosin, five active and five passive residues were selected from actin. Ambiguous interaction restraints identified from these residues was used to drive the docking process, with a maximum effective distance of 8.0 Å (21), which was available for the active-active and active-passive, but not for the passive-passive residues. The best 200 of 1000 refined complexes were obtained from rigid body energy minimization and then submitted to the semi-rigid simulated annealing process. These structures were exposed to a 12-Å shell of TIP3P water solvent for molecular dynamics simulations with a cutoff value of 5 Å. The best 100 complexes were selected according to the evaluation score of their average interaction energies and buried surface area. The HADDOCK score, as the main criteria for selection, was calculated on the basis of the Equation 1,

$$ \text{HADDOCK score} = E_{\text{VDW}} + 0.2E_{\text{ELC}} + 0.1E_{\text{AIR}} + E_{\text{DESO}} \quad (\text{Eq. 1}) $$

where $E_{\text{VDW}}$ is the Van der Waals, $E_{\text{ELC}}$ is the electrostatic, $E_{\text{AIR}}$ is the ambiguous interaction restraint, and $E_{\text{DESO}}$ is the desolvation energy. Finally, optimal candidates with the lowest HADDOCK scores were collected into subsets with more than 10 structures by a backbone-based root mean square deviation clustering with a cut-off value of 7.5 Å. All parameters and processes were carried out through the HADDOCK online service.
Molecular Dynamic Simulations—The module of sander in molecular dynamics package AMBER11 was used for molecular dynamics simulations (22). The prepared crystal structures and the refined actin trimers before docking were relaxed for 20 ns (relMup, relMapo, Dd, and relMapo, sq). After docking, the actomyosin structures were also relaxed by a 100-ns long molecular dynamics, resulting in relA/Mup, relA/Migor, Dd, and relA/Migor, sq. After introducing mutations into the relA/Mup complex, the structures were relaxed for the same length of time leading to relA/Mup,R520Q, relA/Mup,R562Q, and relA/Mup,K622Q/K623Q complexes. All structures were neutralized by adding a discrete number of Na\(^+\) in the most appropriate electronegative areas around proteins. Complexes were then solvated in a truncated octahedron box of TIP3P water with a 12-Å cut-off value along each dimension (23). Long-range electrostatic interactions were treated by particle-mesh Ewald method in periodic boundary conditions (24). By a 1000 steps of the steepest descent and conjugate gradient energy minimization, the compact system was slowly heated up to 300 K and equilibrated at constant temperature (NVT) for 20 ns and then at constant pressure (NPT) for 80 ns. The Berendsen coupling algorithm was used for temperature control (25). The SHAKE algorithm was applied for constraints on covalent bonds and all hydrogen atoms (26).

Binding Free Energy Calculation—Binding free energies of actomyosin complexes from 2500 snapshots of the last 60 ns of the MD simulations were calculated by the molecular mechanic Poisson-Boltzmann surface area method (MM/PBSA) (27). The pbsa module in AMBER was used to evaluate the polar contribution (Gsol-pol) to the solvation free energy (Gsol) (Equation 2).

\[
\Delta G_{\text{sol}} = \Delta G_{\text{sol-pol}} + \Delta G_{\text{sol-np}} \quad (\text{Eq. 2})
\]

### TABLE 1

| Structure          | Mup (1VOM) | Mapo, Dd (1Q5G) | Mapo, sq (2OVK) | EMA/Migor, ch (model) |
|--------------------|------------|-----------------|-----------------|----------------------|
| Relaxed            | relMup     | relMapo, Dd     | relMapo, sq     | No                   |
| Docked             | dockA/Mup  | dockA/Migor, Dd | dockA/Migor, sq | No                   |
| Docked + relaxed   | dockMup    | dockA/Migor, Dd | dockA/Migor, sq | No                   |
| Nucleotide         | ADP-Pi     |                 |                 |                      |

* Structural model of rigor actomyosin based on electron microscopy (EM).

### TABLE 2

| Crystal/model      | Distance*       | Cleft state         |
|--------------------|-----------------|---------------------|
| Mup                | 21.1 ± 1.5      | Open                |
| relMup             | 21.7 ± 1.4      | Open                |
| relA/Mup           | 20.6 ± 1.6      | Open                |
| Mapo, Dd           | 16.7 ± 1.3      | Closed              |
| relMapo, Dd        | 16.7 ± 1.2      | Closed              |
| relA/Mapo, Dd      | 11.9 ± 1.0      | Further closed      |
| Mapo, sq           | 18.3 ± 1.4      | Closed              |
| relMapo, sq        | 18.3 ± 1.3      | Closed              |
| relA/Mapo, sq      | 16.8 ± 1.1      | Further closed      |
| relA/Migor, sq     | 17.2 ± 1.4      | Closed              |

* Calculated on the basis of the distance between the backbone Cα of Glu\(^{365}\) of the upper 50-kDa domain and Asn\(^{537}\) of the lower 50-kDa domain.

Figure 2. The structure of the docked and relaxed rigor actomyosin complexes. Actomyosin structures are fitted into the electron density map (transparent gray) of EMA/Migor, ch with their actin trimers (yellow-red-green) as best fits for the map. Myosin (blue) outside the EM map is colored white. The root mean square deviations of the backbones of dockA/Migor, Dd and relA/Migor, sq from EMA/Migor, ch are 6.0 ± 1.9 and 4.7 ± 1.7 Å, respectively. The root mean square deviations of the backbones of dockA/Migor, sq and relA/Migor, sq from EMA/Migor, ch are 5.8 ± 1.8 and 5.5 ± 1.7 Å, respectively.

Molecular Dynamic Simulations—The module of sander in molecular dynamics package AMBER11 was used for molecular dynamics simulations (22). The prepared crystal structures and the refined actin trimers before docking were relaxed for 20 ns (relMup, relMapo, Dd, and relMapo, sq). After docking, the actomyosin structures were also relaxed by a 100-ns long molecular dynamics, resulting in relA/Mup, relA/Migor, Dd, and relA/Migor, sq. After introducing mutations into the relA/Mup complex, the structures were relaxed for the same length of time leading to relA/Mup,R520Q, relA/Mup,R562Q, and relA/Mup,K622Q/K623Q complexes. All structures were neutralized by adding a discrete number of Na\(^+\) in the most appropriate electronegative areas around proteins. Complexes were then solvated in a truncated octahedron box of TIP3P water with a 12-Å cut-off value along each dimension (23). Long-range electrostatic interactions were treated by particle-mesh Ewald method in periodic boundary conditions (24). By a 1000 steps of the steepest descent and conjugate gradient energy minimization, the compact system was slowly heated up to 300 K and equilibrated at constant temperature (NVT) for 20 ns and then at constant pressure (NPT) for 80 ns. The Berendsen coupling algorithm was used for temperature control (25). The SHAKE algorithm was applied for constraints on covalent bonds and all hydrogen atoms (26).

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The grid spacing of the cubic lattice was set at 2 Å, and the dielectric constant values for the interior and exterior of the system were 1 and 80. The non-polar contribution to the solvation free energy (ΔG_{sol-np}) was calculated from the solvent-accessible surface area (Equation 3) (28).

\[ \Delta G_{sol-np} = \gamma \text{SASA} + \beta \]
(Eq. 3)

The surface tension γ and the offset β were set to 0.00542 kcal mol^{-1} Å^{-1} and 0.92 kcal mol^{-1}, respectively. The contribution of entropy (−TΔS) based on ligand receptor association was performed with normal-mode analysis (29). 1000 snapshots were collected and submitted to molecular minimization with a distance-dependent dielectric constant, E = 4r. The end of convergence was not achieved until the root mean square deviations of the gradient vector were less than 1 × 10^{-4} kcal mol^{-1} Å^{-1}. Residues close to the binding surface with a distance less than 20 Å were acquired to estimate the contribution of entropy.

**RESULTS**

After completing the actin trimer (17) and the different myosin 2 structures (Table 1) with their missing residues, they were relaxed by molecular dynamics and docked to each other using the HADDOCK program. The docked structural models and the uncomplexed actin and myosin structures were placed into explicit water boxes and relaxed by a 100-ns long molecular dynamics simulation run.

**TABLE 3**

Weak and strong binding of the relaxed actomyosin complexes

Binding free energy is calculated on the basis of ΔG_{bind} = ΔH − TΔS. K_d values are calculated on the basis of ΔG_{bind} = −RTlnK. SIM = simulated, EXP = experimentally determined values.

| structure       | ΔG_{bind} (kcal/mol) | ΔH (kcal/mol) | ΔS (cal/K/mol) | ΔG_{sol-pol} (kcal/mol) | ΔG_{sol-np} (kcal/mol) |
|-----------------|----------------------|---------------|----------------|--------------------------|--------------------------|
| A-M_Apo         | -7.6±0.8             | 2.8           | -5.4           | 26                       |
| A-M_Apo,In     | -12.4±1.3            | 0.013         | -9.6           | 0.03                     |
| A-M_Apo,Ex     | -11.4±1.0            | 0.007         | -9.2           | 0.19                     |

**TABLE 4**

Interactions of actomyosin complexes

Interactions present in both open and closed actin-binding cleft states of myosin are bolded. All listed interactions have >75% occurrence during molecular dynamics.

| actin-binding loops | type of interaction | open actin-binding cleft state | closed actin-binding cleft states |
|---------------------|---------------------|--------------------------------|----------------------------------|
|                     |                     | relA-M_{Apo,myosin} - actin   | relA-M_{Apo,rigor,2d} - myosin - actin | relA-M_{Apo,rigor,eq} - myosin - actin | relA-M_{Apo,rigor,ch} - myosin - actin |
| Loop 2              | salt bridges       | Lys622 – Asp25               | Lys622 – Asp24/Asp25/Lys635 – Asp1 | Lys640 – Asp25/Asp3/Glu4 | Glu629 – Arg28/Lys640 – Asp25/Lys642 – Glu3|
|                     | H-bonds            | Ser641 – Asp24               | Ala642 – Asp24                   | Ser643 – Asp25/Lys652/657 – Glu24/Lys660 – Glu1/2 | Gly635 – Asp24/Lys637 – Gly23/Ser348/Lys638 – Gly23/Lys642 – Glu34/Lys643 – Ser145 |
|                     | salt bridges       | Glu365 – Lys328              | Glu365 – Arg147                  | Glu373 – Arg147          | Arg371 – Glu372/Lys328/Gln314/Ile329/Glu373 – Arg147 |
|                     | H-bonds            | Arg402 – Glu334              | Arg402 – Glu93                   | Gly401 – Pro27/Gly407 – Glu334/Leu399 – Arg28 | Gly415 – Glu334/Gly416 – Glu334/Lys407 – Asp25 | Gly411 – Lys336 |
| CM loop             | H-bonds            | Gly401 – Pro27               | Gly415 – Glu334                  | Gly416 – Glu334/Gly407 – Asp25 | Gly411 – Lys336 |
|                     | salt bridges       | Arg520 – Asp1                | Arg520 – Asp3/Glu4               | Lys528 – Asp1/Glu4       | Arg520 – Asp1/Glu4/Lys528 – Asp1/Glu4 |
|                     | H-bonds            | Gly521 – Gln534              | H-bonds                          | Lys544 – Gly48/Lys552 – Asp52 – Gln49/Gly553 – Gly46/Gly48 | Ser549 – Gln49/Lys552 – Gly48/Lys553 – Gly46/Gly48 |
| Activation loop     |                     | H-bonds                        |                                |                          |                                |                                |
|                     |                     | Arg520 – Asp1                | Arg520 – Asp3/Glu4               | Lys528 – Asp1/Glu4       | Arg520 – Asp1/Glu4/Lys528 – Asp1/Glu4 |
| lower 50 kDa domain |                     |                                |                                |                          |                                |                                |
| Loop 3              | salt bridges       | Arg562 – Glu99               | Arg562 – Glu99                   | Lys567 – Asn92/Lys569 – Glu99 | Lys567 – Glu99/Lys572 – Asp1/Glu2/Lys572 – Asp1/Glu2/Lys572 – Glu100/Glu577 – Arg95 | Lys567 – Glu99/Lys572 – Asp1/Glu2/Lys572 – Asp1/Glu2/Lys572 – Glu100/Glu577 – Arg95 |
|                     | H-bonds            | Ser564 – Arg95               | Lys565 – Tyr91                   | Gly567 – Arg95           | Lys567 – Glu99/Lys572 – Asp1/Glu2/Lys572 – Asp1/Glu2/Lys572 – Glu100/Glu577 – Arg95 | Lys567 – Glu99/Lys572 – Asp1/Glu2/Lys572 – Asp1/Glu2/Lys572 – Glu100/Glu577 – Arg95 |

1 Van der Waals contribution.
2 Electrostatic energy contribution.
3 Polar contribution to the solvation free energy (Poisson-Boltzmann surface area).
4 Non-polar contribution to the solvation free energy based on the solvent-accessible surface area.
5 Enthalpic contribution based on ΔH = ΔE_{vle} + ΔE_{ele} + ΔG_{sol-pol} + ΔG_{sol-np}.
6 Entropic contribution based on the normal mode analysis.
Actin induced the closure of the actin-binding cleft of the non-actin-bound Mapo1, whereas loop 4 (ice blue), cardiomyopathy loop (purple), and loop 2 (ice blue) of the upper 50-kDa domain do not differ significantly (SIM) from the weak, relA Mrigor,sq complexes.

In the weak actin-binding state (relA Mrigor,sq), actin-binding clefts of the non-actin-bound Mapo1, dockA Mrigor,ch, and dockA Mrigor,sq did not match well with the EMA Mrigor,sq structures (Fig. 3 and further closed cleft states of Table 2) for all actomyosin interactions (Table 4). This interaction was not described in the in silico model, interaction was not established in the protein-protein docking procedure, whereas it was formed spontaneously in the first few nanoseconds of the molecular dynamics relaxations. In the EMA Mrigor,ch model, this interaction was not described because the N-terminal region of actin was missing from the actin crystal structure used for creating the model. In the weak actin-binding state, conformation of the activation loop is different from that of the rigor. Arg520 in relA Mrigor it forms a more extended salt bridge cluster with Asp3 and Glu4 (Fig. 3, Table 4). These interactions are stable as their occurrence is 95% throughout the whole molecular dynamics. Loop 2 goes through a large conformational change during the weak to strong actin-binding transition. In both states, there is a salt bridge between myosin and actin.
Lys622 and actin Asp25, whereas the C-terminal part of loop 2 forms a salt bridge with the actin N-terminal peptide in rigor.

The helix-loop-helix element (residues 530–537), structurally coupled to the activation loop, has stronger hydrophobic interactions with residues 143–147 of actin in rigor compared with the weak binding state. These results are in agreement with previous indications that hydrophobic interactions create expanding networks of the actin-myosin interface during the weak to strong actin-binding transition (32). Furthermore, a conformational rearrangement of the interaction network occurs between the activation loop and the actin N-terminal region upon the up-to-down lever movement and the weak to strong actin-binding transition, which indicates that actin may directly affect the relay/converter/lever movement through their structural coupling to the activation loop.

Upon molecular dynamics of dockA: Mup, significant conformational relaxations occurred in the relay/converter and the nucleotide-binding region. All conformational relaxations were completed in the first 10 ns and remained stable in the following 90 ns of the simulations. The most striking effect induced by actin binding was an 18° further up movement of the myosin lever (further up-lever state of relA Mup in Table 5). The further up-lever movement occurred within 5 ns of the relaxation and remained stable in the following 95 ns (Fig. 4, A and B, left panel). To investigate the role of the activation loop-actin interaction in this effect, Arg520 of relA Mup was mutated in silico to Gln (relA Mup R520Q) (supplemental file relA Mup R520Q.pdb). This mutation interrupts the salt bridge interaction between the activation loop and the actin N-terminal region. The mutated construct was relaxed by molecular dynamics for 100 ns. In the first 5 ns the lever moved back to the same up position as that the actin detached myosin (relMup). This specific effect on the extra priming caused by the activation loop-actin interaction is supported by the fact that mutations in other actin-binding regions (K622Q/K623Q in loop 2 and R562Q in loop 3) caused only slight “back” relaxations and the lever remained mainly in the further up position (Fig. 4B, left panel, and Fig. 5A, relA Mup K622Q/K623Q and relA Mup R562Q in Table 5) (supplemental file relA Mup R562Q.pdb).

As expected, the further up-lever movement was coupled to another conformational relaxation that occurred in the nucleotide binding pocket (Table 5). As a result, the C-terminal part of the switch 2 loop in relA Mup possesses a further closed position (Fig. 4A). Similarly to the lever relaxation, when Arg520 of the activation loop was mutated to Gln (relA Mup R520Q), the switch 2 loop relaxed to the same closed position as that of the actin detached, closed conformation (relMup), whereas in loop 3 and loop 2 mutants (relA Mup R562Q and relA Mup K622Q/K623Q) switch 2 remained in the further closed position (Fig. 4B, right panel, and Table 5) without any back relaxations. The effect of

| Angle (°) | Lever state | Switch 2–switch 1 distance | Switch 2 position |
|----------|-------------|---------------------------|------------------|
| Mup (1VOM) | 122 | Up | 10.4 | Closed |
| relA Mup | 123 ± 3 | Up | 10.3 ± 0.2 | Closed |
| relA Mup R520Q | 105 ± 3 | Further up | 9.5 ± 0.2 | Further closed |
| relA Mup R562Q | 120 ± 3 | Up | 10.2 ± 0.2 | Closed |
| relA Mup K622Q/K623Q | 111 ± 3 | Further up | 9.5 ± 0.2 | Further closed |
| relA Mup | 113 ± 2 | Further up | 9.6 ± 0.2 | Further closed |

FIGURE 4. The extra primed state of the weak binding actomyosin complex. A, after docking and relaxation of myosin (gray) and actin (green-yellow), the relA Mup (red) possesses a further up-lever and a further closed switch 2 compared with relMup (cyan) and the overlaid PDB 1MMD down-lever motor domain (orange) structures. Arbitrary extensions of the levers are represented by red, cyan, and orange cylinders, respectively. Inset, positions of switch 2 and switch 1 in further up (red) and up (cyan) states of myosin. B, changes in the angle of the position of the lever (left panel) and the distance between switch 1 and switch 2 (right panel) throughout the molecular dynamic simulations of A Mup (red), relA Mup R520Q (blue), relA Mup R562Q (green), and relA Mup K622Q/K623Q (pink), and as a control, Mup (black), R250Q, R562Q, and K622Q/K623Q mutations interrupt the interactions of actin with activation loop, loop 3, and loop 2, respectively.

TABLE 5
Converter/lever and switch 2 positions of prepowerstroke myosin and weak binding actomyosin complexes upon molecular dynamics

Angles were measured between the Cα atoms of Gly684 and Arg520 of SH1 helix and Ala748 of the converter domain.

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mutation in the activation loop indicates that the activation loop has a key role in the extra priming of myosin. The structural changes in myosin caused by actin binding are transmitted from the activation loop to the nucleotide binding pocket and relay region through the wedge loop. Upon actin binding, the wedge loop forms interactions with the lower 50-kDa helix-loop-helix (upper relay) region, the relay helix, and the C-terminal region of switch 2 \((\text{relMup and relA-M}_{\text{up}} \text{ of Fig. 5})\), thereby structurally connecting these regions in the weak actin-binding state. This interconnection between the upper relay and switch 2 does not exist in the rigor state and in the absence of actin. When a salt bridge is formed between Asp\(^1\) of actin and Arg\(^{520}\) of the activation loop in the weak actomyosin state, a hydrophobic cluster \((\text{cloud})\) is formed between the upper relay, wedge loop, and switch 2. The newly formed interaction pulls switch 2 to its further closed position. The relay helix is reoriented and stabilized, resulting in the further up position of the lever by the Leu\(^{529}\)-Trp\(^{584}\)-Phe\(^{466}\) hydrophobic cluster and the Glu\(^{580}\)-Lys\(^{462}\) salt bridge. This reorientation of switch 2 and the relay does not occur in the activation loop mutant \((\text{relA-M}_{\text{up}, R520Q} \text{ of Fig. 5})\), whereas they remain in the same orientation in loop 3 and loop 2 mutants \((\text{relA-M}_{\text{up}, R562Q} \text{ and } \text{relA-M}_{\text{up}, K622Q/K623Q} \text{ of Fig. 5})\). These results indicate that the activation loop is a specific sensor of actin binding and is responsible for transferring the information of actin binding to the nucleotide binding pocket and the relay/converter region.

We analyzed changes of the motional correlations of the functional regions of myosin upon actin binding. Positive motional correlation between the two regions is defined as their coupled dynamics is high. In the absence of actin \((\text{relMup and relA-M}_{\text{up}})\) the dynamics of switch 2 is weekly coupled to other functional regions of myosin (Fig. 6). The dynamic coupling between switch 2 and the relay is highly increased in the weakly and strongly actin-bound states \((\text{relA-M}_{\text{up}} \text{ and relA-M}_{\text{rigor, Del}})\). The dynamic coupling becomes very high between switch 2 and the upper relay, including the activation loop, the relay helix, and the wedge loop. When the salt bridge interaction between the activation loop and actin was interrupted by the R520Q mutation, the motional correlations between these regions decreased to the same level as that of the actin detached states \((\text{relA-M}_{\text{up}, R520Q})\). Mutations in loops 3 and 2 did not decrease the motional correlation of switch 2 to the level of the actin detached states \((\text{relA-M}_{\text{up}, R562Q} \text{ and } \text{relA-M}_{\text{up}, K622Q/K623Q})\). These effects picture the same communication pathways found by the analysis of the structural rearrangements described above.

**DISCUSSION**

Recently, the mechanism of the powerstroke has been proposed on the basis of crystal structures and pseudoatomic cryo-EM models (7–9). However, neither the starting nor the end states of the powerstroke structures of actomyosin are available. Generally, the actin detached, up-lever myosin head complexed with an ADP-P, analog (PDB codes 1MMD, 1VOM, and 2V26) is discussed as the starting state of the powerstroke (33). However, one may assume that an actin-detached state is not the initial state of the powerstroke, because an effective powerstroke must start in an actin-at-tached state (4). Also, the electron micrograph rigor structures (7–9) cannot be considered as part of the powerstroke, because the powerstroke ends in a nucleotide-bound state (34).
Actin-induced conformational changes in the M-up/ADP-P state were speculated based on comparison of the rigor cryo-EM model and the actin-detached M-up/ADP-VO₄ crystal structures (7, 8). Actin-binding cleft closure was considered to be the major actin-induced conformational change that was supposed to initiate rearrangements in the nucleotide binding pocket leading to the powerstroke. To overcome the problem of the missing structure of the initial state of the actomyosin prepowerstroke, in this study we constructed the atomic model of the weak actin-binding state (A-M-up/ADP-P).

We demonstrate that the lower 50-kDa subdomain, including loop 3, H-loop, and the activation loop, is the major contributor for the initial, weak actin binding of myosin. We also confirm the interaction of loop 3 with two actin monomers (34, 35), which are among the initial weak actin-binding interactions. Structural and mutational studies indicate that dynamic interactions of loop 2 with different regions of actin in the different states of actomyosin reflect its role in the weak to strong actin-binding transition of myosin (36, 37). We found that loop 2 positioned differently in rigor compared with the weak actin-binding state. As actin binding strengthens, loop 2 starts to compete with the activation loop for the highly negatively charged N-terminal region of actin, while also still interacting with other regions of actin (Asp²⁴/²⁵). Similarly to our results, a recently published in silico structural study also demonstrates that the activation loop and loop 2 interact with the actin N-terminal region in rigor actomyosin (38). Within the first 15 ns of their molecular dynamic simulation, the activation loop forms stable interactions with Glu² and Asp³, whereas it competes with loop 2 for Asp¹ of actin. In rigor actomyosin a similar
“sandwich-like” pattern (actin Asp⁶⁴/Asp⁶⁵-myosin loop 2-ac- tin N-terminal region-myosin activation loop) was found in the rigor actin-myosinE complex (8). Former rigor models lack the interaction of the activation loop with actin, because of the missing N-terminal region of the applied actin structures (7, 10), although this interaction is crucial for actin activation of myosin (6).

We found that actin binding causes a structural relaxation of the myosin structure. We note that conformational relaxations are not hindered by free energy barriers, and thus occur at the nanosecond time scale, therefore they must be distinguished from first-order kinetic steps. The major structural rearrange- ments occur in the relay/converter/lever region and the switch 2 loop: an 18° further up-lever movement is coupled to a further closure of the switch 2 loop. A minor actin-binding cleft closure was also detected upon molecular dynamics relaxation. We demonstrated that the activation loop has an essential role in the extra priming of myosin, because the actin-induced confor- mational changes are transmitted through the activation loop toward the relay and active sites. This structural finding is in accordance with recent experimental evidence that the activation loop is specifically responsible for actin activation of the ATPase activity of myosin by accelerating the up-to-down lever movement (6).

Irrespective of the lever swing occurring in ADP-P₁ or ADP states of myosin, the initial state of the powerstroke is undoubt- edly the open actin-binding cleft ρM₂ state, which weakly binds to actin. Intriguingly, myosin 6 complexed with ADP-VO₄ (PDB 2V26) (39) is highly similar to the presented structure of ρM₂ possessing a further up-lever and a further closed switch 2 (Fig. 7). The existence of such a state demonstrates that it is a structurally accessible state of myosin.

Here, we determined the relaxed conformational structure of the weak actomyosin complex in the prepowerstroke state. Its significance is that the powerstroke (including actin-binding cleft closure, switch movements and lever swing) starts from this initial state. The presented ρM₂ structure may lead to further experiments to reveal a more detailed reaction coordinate pathway of the powerstroke.

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FIGURE 7. Comparison of the extra primed state in myosin 2 and myosin 6 and their structural transmission pathways in the absence of actin. A, activation loop, switch 2, the relay and the converter region are highlighted from myosin (gray). Myosin 6 prepowerstroke state (PDB 2V26, blue) possesses a similar, or even more further primed lever and further closed switch 2 than ρM₂ (red). The two myosin structures were aligned by their backbones. B, in the ρM₂ structure, β-sheet (orange), switch 2 (pink), and the relay (blue) are not connected through the wedge loop (gray) with the upper relay (yellow) and activation loop (red). In the structure of myosin 6 (PDB 2V26), switch 2 creates interactions with the wedge loop, but the wedge loop has no further interactions with the upper relay region.
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