Abstract

Myosin VI is a molecular motor that can walk processively on actin filaments with a 36 nm step size. The walking mechanism of myosin VI is controversial because it takes very large steps without an apparent lever arm of required length[35]. Therefore, myosin VI has been argued to be the first exception to the widely established lever arm theory. It was therefore critical to directly demonstrate whether this motor walks hand-over-hand along actin in spite of its short lever arm. In the present work the displacement of a single myosin VI head was followed during the stepping process. A single head is displaced 72 nm during stepping[66], while the center of mass previously has been shown to move 36 nm[49,74]. Thus, this result provides strong evidence for a hand-over-hand walking mechanism. The existence of a flexible element is hypothesized that would allow the motor to bridge the observed 72 nm distance.

We established a new technique termed single molecule high resolution colocalization (SHREC) that allows the measurement of interfluorophore distances below the diffraction limit of the fluorophore's emitted light[67]. To this end, two chromatically different fluorescent dyes were used as probes. The probes were imaged separately, and their centroids were localized individually with nanometer precision. Subsequently, the fluorophores' positions were mapped onto the same space, which allowed the determination of the distance between them. With a lower resolution limit of ~10 nm, SHREC is a tool that can measure distances at scales between the upper resolution limit of single molecule FRET (~10 nm) and the lower resolution limit of fluorescence microscopy (~250 nm). The capability of SHREC was tested using the processive myosin V molecular motor. With its lever arms, myosin V walks hand-over-hand along actin. This mechanism predicts, similar to myosin VI, an alternation of the catalytic heads which we directly visualized with SHREC by labeling a motor's two lever arms with two different fluorophores. As predicted by the hand-over-hand model, we observed the fluorophores alternate positions as the motor walked along actin.

The actin activated ATPase activity of full-length *Dictyostelium* myosin II is stimulated 6-fold upon reversible phosphorylation of its regulatory light chain (RLC)[17]. In contrast, the ATPase of the single headed S1 is activated regardless of the phosphorylation state of the RLC. The molecular mechanism of the regulation has remained unclear because the RLC is topographically far removed from the catalytic domain in available crystal structures[7,10]. Unexpectedly, we observed the RLC crosslink to the catalytic domain in the single headed *Dictyostelium* myosin S1, suggesting an interaction between the RLC and S1. We also observed that phosphorylation of the RLC inhibited this crosslinking. The increased interactions between the head and the neck in the unphosphorylated state suggests a more "bent" conformation of the protein, as was seen in the unphosphorylated smooth muscle myosin II in electron microscopy experiments[24,25]. The phosphorylation state dependent conformational change in the *Dictyostelium* Myosin S1 combined with previous structural information suggests a model for the regulation of the actin activated ATPase activity. In this model, dephosphorylation of the RLC favors a conformation in which the head cannot bind actin productively due to steric hindrance in the context of the full-length molecule.