Myosin VI Lever Arm Rotation: Fixed or Variable?

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Abstract: Two recent articles[1,2] addressed the power-stroke of myosin VI molecules during stepping. Although both groups measured the angles of fluorescent probes attached on the myosin VI molecule lever arm using polarized fluorescence techniques, they differ about whether the myosin VI lever arm rotation is fixed[1] or variable[2]. Here we discuss the causes of the discrepancy between the two studies and the implications for myosin VI processive motility.

Introduction: Myosin VI, an unconventional myosin motor that walks toward the minus ends of actin filaments, has received great attention recently. Reifenberger et al.[1] have presented evidence from Defocused Orientation and Positional Imaging (DOPI) assays that myosin VI lever arms tilt by an angle very close to 180° on each step. This contrasts with results from Sun et al.[2] wherein we applied single molecule Polarized Total Internal Reflection Fluorescence (polTIRF) microscopy and found variable degrees of tilting both in the axial and azimuthal directions relative to actin. This variability is consistent with the known variable step size of myosin VI. There are several technical differences in the two papers that interested researchers should understand, and more importantly, substantive differences in the conclusions.

Results and Discussion: Reifenberger et al.[1] explained the difference in results between the two studies by a choice of analysis hemisphere, but their explanation is wrong. Fig. 1, here, shows lateral and axial views of a myosin molecule (M) that landed on actin (A) in a typical position away from the coverslip (blue). Although the fluorescence emission from the chromophore is symmetric about the dipole axis, the two ends of the bifunctional probe are different because they are linked to different cysteine residues (here 66 and 73 on calmodulin, in the same construct used by both studies). To indicate this asymmetry we draw the probe as a directional arrow. Neither DOPI nor polTIRF can intrinsically distinguish between the two ends of the dipole, so the choice of which one to report requires additional considerations. It is especially crucial to be sure that it's the same one before and after an angle change (e.g., after a motor step). For any measurement of polarized fluorescence, a hemisphere needs to be chosen that defines the range of valid angles reported. Fig. 1A shows the position of the probe before a step. Follow the pointed end of the arrow in Fig. 1 which is at the Cys^{66} end of the probe. If the lever arm rotates 180° axially (in a plane containing the actin), then the probe will be positioned as in Fig. 1B after the step. With an analysis hemisphere whose equator is parallel to the glass, as used by Reifenberger et al.[1], the pointed end of the probe is positioned inside the hemisphere before (Fig. 1A middle panel) and outside the hemisphere after the step (Fig. 1B middle panel). In the latter case, the method will report the wrong angle. If the hemisphere is tilted around the axis of actin, as we did[2], then the relevant end of the dipole is reported correctly before (Fig. 1A lower panel) and after (Fig. 1B lower panel) the step. This idea was explained in our original paper on DOPI (Suppl. Fig. 5)[3] and formally shows the error in Reifenberger et al.'s categorical claim that a horizontal hemisphere should always be used.

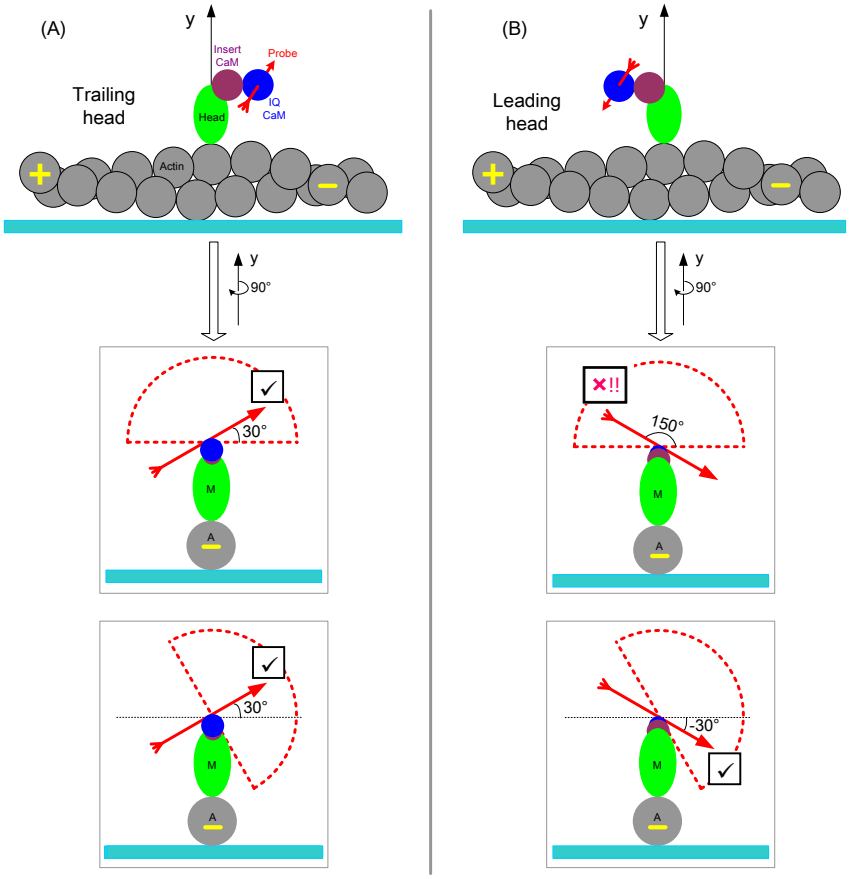

Figure 1. Views of myosin VI in the trailing (A) and leading (B) positions. In the upper panels of A, side views show the rigor state and bifunctional rhodamine bound to calmodulin residues 66 and 73. The rhodamine dipole is marked as a directional arrow to identify the end attached to Cys[66]. Myosin VI steps toward the "–" (pointed) end of actin, drawn to the right here. Note that the results of Sun et al.[2] show that the rotation is variable, but for illustrative purposes, only the 180° rotated angle is shown in B. Middle and lower panels show an axial view from the "–" end of actin, looking toward the "+" end. The middle panels indicate what angles are reported if a horizontal hemisphere is used to select the range of allowable probe angles, as used by Reifenberger et al.[1] In the trailing head, this hemisphere contains the Cys[66] end of the dipole, but in the leading head, the opposite end of the dipole is within the hemisphere (B, middle panel), causing an erroneous angle to be reported [**X!!**]. The bottom panels show that a tilted hemisphere reports the correct angle (i.e., the same end of the dipole) in both leading and trailing positions. Thus, the analysis hemisphere cannot be horizontal for all molecules to obtain the correct angle from the DOPI or polTIRF methods.

The same point is shown in Fig. 3 of Reifenberger et al.[1] (red and orange arrowheads). Molecules are also described in Reifenberger's PhD thesis[4] (pp. 138 – 143, Figs. 5.21 – 5.24) that artifactually jumped between the upper and lower hemispheres. A slight tilt of the equator of the analysis hemisphere would suppress this artifact, again showing that the correct hemisphere is not always horizontal.

Myosin VI molecules are shown to "wobble" on p. 136 of Reifenberger's thesis[4]. That this point was omitted from the publication[1] is surprising, inasmuch as it was a major conclusion of Sun et al[2].

Reifenberger et al. point out that the 180° position of the leading head lever arm is compatible with the pre-powerstroke x-ray crystal structure containing ADP and a P_i analog[5]. However, P_i and probably ADP are both released rapidly from the leading head upon binding to actin[6], so the crystal structure with both products bound is irrelevant in the present context.

It is not possible to gauge from Reifenberger et al.'s paper[1] whether their probe molecules were cross-linked between the two engineered Cys residues and thereby were constrained fully to lie along the axis between the two target Cys residues. The DOPI technique has the advantage over polTIRF of simultaneously monitoring position and stepping, but this is offset by the disadvantage that it doesn't provide an independent estimate of probe wobble. polTIRF does report the wobble, both on the nanosecond and microsecond timescales, so we can be sure that all of the molecules analyzed in Sun et al. were immobilized on the calmodulin. Whether this accounts for some of the differences in the results is not clear at present.

Myosin VI is known to have a broad distribution of step sizes giving a 25 to 40 nm span between the two heads. This point is confirmed by ours and earlier data[7-9]. Thus, the geometrical relationship between the two lever arms should depend on the distance between the two heads after any given step, a point made previously by both groups[2,9]. In Reifenberfer et al.[1], the variable step size is dismissed by postulating that each molecule walks straight, rarely taking sideways steps, and the overall distance distribution is broad due to contributions from different molecules. This argument is fallacious for at least four reasons: a) if each molecule has a fixed step size, then certain molecules would need short steps and others long ones, both of these groups of molecules exhibiting strong helical (not straight) motion[10,11]; b) All individual recordings presented in papers from the authors' lab[1,4,9] and others[7,12] show variable steps; c) Binding sites for myosin VI to a filament on the glass allow a broad distribution of azimuths (α) (Fig. 3 of Sun et al.[2]), but the α distributions (Fig. 2 of Reifenberger et al.[1]) show narrow peaks. This result implies severe restriction of myosin VI's landing positions or else strongly biased selection of recordings; and d) Myosin V has been shown sometimes to take sideways steps[13,14]. If Reifenberger et al's argument were correct, then step size distributions of myosin V for individual molecules would be wide, but they are narrow. Thus, negating the previous data showing the variable step size of individual myosin VI molecules on the basis of Reifenberger et al.'s DOPI results is untenable.

If we retain the well established variable step size, and consider Reifenberger et al.'s proposal that the leading head lever arm is completely decoupled from the converter domain, it is difficult to understand how its angle, relative to actin, can be fixed at 180° independent of the (variable) distance between the two heads. A mechanical model with flexible connections shows that the relative lever arm angles depend on the distance between the heads (Sun et al.[2], Fig. 6). The azimuthal orientation between the two bound heads is determined by the actin helix, so it also has to vary with step size, as we found.

Nevertheless, both studies conclude that the lever arm of myosin VI is not tightly coupled to the converter domain. Reifenberger et al. suggest that the lever and converter are completely uncoupled in the leading head of a walking myosin VI molecule. But, contradictorily, strain in the molecule is supposed by Reifenberger et al. to prevent the leading head from binding ATP in order to maintain processivity. This strain-gating conjecture is incompatible with complete lever arm-converter uncoupling, which would allow the leading head converter domain to rotate fully and rapidly to its rigor position, thereby posing no restraint on ATP binding. Although Reifenberger et al. do not explain how converter rotation is limited or how the 180° angle is maintained, they hint that the lever arm is tethered between two flexible hingepoints, the N-terminal hinge between the lever and the converter (the point of "complete uncoupling") and the C-

terminal hinge extending to an elastic segment, perhaps, for example, an unfolded 3-helix bundle. Linear strain (no torque) would then prevent ATP binding until the trailing head detaches. In that case, the lever would undergo large thermal rotational fluctuations about its own axis and the dipole at Cys^{66}-Cys^{73} would exhibit much larger angular fluctuations in the leading than in the trailing position. Neither Reifenberger et al.'s nor our data shows signs of such extensive azimuthal wobble in the leading head.

Conclusions: We conclude, as originally discussed in Sun et al.[2], that a pliant region between the lever arm and converter (that is, not complete uncoupling), similar to that found by x-ray crystallography of scallop myosin[15] enables partial rotation of the leading head converter domain. Torque on the converter would prevent full rotation and ATP binding until the trailing head detaches. The variable angle of the lever arm that we observed is set by the geometrical relationship of the two bound heads and partitioning of the total compliance between this rotational spring and the other flexible regions in the molecule.

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