Simultaneous Measurement of Nucleotide Occupancy and Mechanical Displacement in Myosin-V, a Processive Molecular Motor

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ABSTRACT   Adenosine triphosphate (ATP) turnover drives various processive molecular motors and adenosine diphosphate (ADP) release is a principal transition in this cycle. Biochemical and single molecule mechanical studies have led to a model in which a slow ADP release step contributes to the processivity of myosin-V. To test the relationship between force generation and ADP release, we utilized optical trapping nanometry and single molecule total internal reflection fluorescence imaging for simultaneous and direct observation of both processes in myosin-V. We found that ADP was released 69 ± 5.3 ms after force generation and displacement of actin, providing direct evidence for slow ADP release. As proposed by several previous studies, this slow ADP release probably ensures processivity by prolonging the strong actomyosin state in the ATP turnover cycle.

Myosin-Va (henceforth myosin-V) is the most commonly studied processive motor (1–3). By forming a homodimer, myosin-V transports cargos over long distances requiring many adenosine triphosphate (ATP) turnover cycles without detaching from the actin-filament. During these cycles, ATP binds to myosin-V and is hydrolyzed to adenosine diphosphate (ADP), which is then released. Although ADP release is thought to contribute to the myosin-V motility, there is no direct evidence affirming the relationship between ADP release and force generation (4–6). Measurements of ADP release, Pi-release, and Pyrene-actin fluorescence in biochemical studies have indicated a relationship between ADP-release and Pi-release or the weak to strong transition in the actomyosin binding state (7–9). However, these studies could only speculate on the relationship between ADP release and force generation or mechanical displacement between myosin and actin. Meanwhile, mechanical studies of myosin-V could observe the timing of the force generation, but they could not directly observe the ADP release step (4,5). Therefore, there is no direct experimental observation of the relationship between ADP release and force generation. In this letter, we attempted to clarify the timing of these events and resolve the role of ADP release in myosin-V based motility.

We recently constructed a novel system to simultaneously measure occupancy of the active site by fluorescent nucleotides and force generation in individual single-headed myosin-Va Subfragment 1 IQ6 (henceforth myosin-V S1) molecules (10). The setup is based on a previous method with some modifications (see Fig. S1 and additional text in the Supplementary Material) (10,11). Myosin-V S1 was fixed to a 1-μm-wide glass pedestal through a Myc-tag bound to an anti-Myc antibody adhered to the glass surface via protein L. Briefly, displacement of actin by myosin-V S1 was measured using optical trapping nanometry, while individual ATP turnover cycles were visualized using prism-

type total internal reflection fluorescence-microscopy and a fluorescent-ATP analog 2′-O-Cy3-EDA-ATP (Cy3-ATP) (12). Cy3-ATP was found to support robust motility of myosin-V in gliding filament assays and single molecule measurements (see supplementary text, Table S1, Fig. S3, and Fig. S4). It was shown in a previous report that in our simultaneous observations, the mechanical record is derived from the same molecule as the ATP-analog binding signal (10).

Fig. 1 (upper trace) shows the time-course displacements by myosin-V S1 of a bead-actin-bead dumbbell held in the optical trap. The rising and falling phases correspond to the force generation of myosin-V S1 and its detachment from the actin filament, respectively. Fig. 1 (bottom trace) shows the time course of the fluorescence intensity changes of the Cy3-nucleotide. Fluorescent-spots were observed coupled to the mechanical cycles (solid arrow). Since the fluorescent spots appeared at almost the same time as the falling-phase of the displacement, these events were attributed to Cy3-ATP-binding to myosin-V S1. The fluorescent spots disappeared following the subsequent rising phase of the displacement. Since force-generation is unlikely to be coupled to ATP release and Pi dissociates rapidly from acto-myosin-V-ADP-Pi (7,8,13), the reduction of intensity was attributed to release of the Cy3-ADP from acto-myosin-V-Cy3-ADP. Some displacements (Fig. 1, open arrows) did not couple to
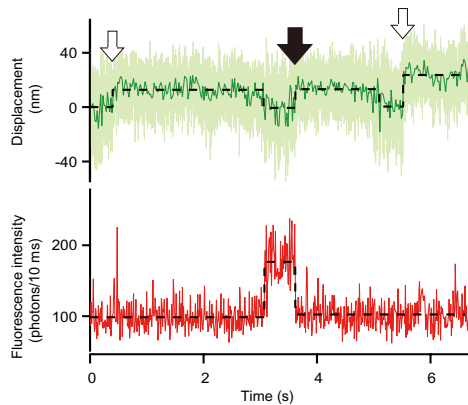
FIGURE 1 **Simultaneous observation of ATP-turnover cycles and force generation by myosin-V S1. The upper trace shows the time course of the trapped bead displacements. Raw data and data passed through a 100 Hz low-pass filter are superimposed. The bottom trace shows the fluorescence intensity change of a Cy3-nucleotide located at the myosin-V position. Open arrows show mechanical events coupled to binding of nonfluorescent ATP. The closed arrow shows an event coupled to binding of Cy3-ATP.**

detection of fluorescent-spots, suggesting that these events likely accompanied binding of nonfluorescent ATP. The fraction of mechanical events coupled to Cy3-nucleotide binding was 2.5%. The ratio of ATP (1 μM) to Cy3-ATP (100 nM) in the medium and the lower affinity of Cy3-ATP for actomyosin-V (Table S1) accounts for the observed proportion of Cy3-associated mechanical events. This result is consistent with a recent report showing 1:1 coupling between stepping of double-headed myosin V and binding of 3′-(7-diethylaminocoumarin-3-carbonylamino)-3′-deoxyATP (14).

ATP-binding to myosin-V S1 occurred concomitantly with the falling-phase of the displacement, indicating Cy3-ATP induced detachment of myosin-V S1 from actin (Fig. 2 a). The distribution of delay times between ATP binding and myosin-V S1 detachment, which were determined respectively using a stepfinder MatLab (The MathWorks, Natick, MA) routine kindly provided by Drs. J. Kerssemakers and M. Dogterom (15), was fitted by a Gaussian function with a mean ± SD of 6.0 ± 31 ms ($n = 61$) (Fig. 2 b). The deviation indicates the limit of resolution for effectively detecting the event times (11). Thus, we conclude myosin-V S1 detachment occurred promptly upon ATP binding.

ADP release always occurred following force generation after a delay (Fig. 2 c). The delay time distribution was fitted by a single exponential function with a mean rate constant mean ± SE of 14 ± 1.1 s^{-1} ($n = 61$), indicating ADP is released from myosin-V in a first-order process (Fig. 2 d). This value agrees with several biochemical studies reporting an ADP-release rate from the actomyosin-V-ADP complex of 12–22 s^{-1} (7,16). The same studies also suggested that myosin-V spends the majority of its ATPase cycle bound in the acto-myosin V-ADP state. Other studies have shown that ADP release follows Pi-release (8,9,13). Thus, the ob-

served ADP release after force generation corresponds to the elementary ADP release process from actomyosin-V-ADP, not the ADP-Pi complex. This is the first direct evidence of slow ADP release after force generation. Furthermore, since the ADP-bound state is considered to be a strong actomyosin state, the delay of ADP release stabilizes actomyosin binding by prolonging the strongly bound state and thus probably improves myosin V processivity.

Recently, the ADP-release rate was found to be strongly affected by applied force (4–6). According to the proposed relationship between ADP-release rate and force (4), the zero-load ADP-release rate of 12–22 s^{-1} would decrease to 9–16 s^{-1} at 0.34 pN, the average force applied in our experiments (supplementary text). However, this force level is too small to make conclusions about the force dependence of ADP release from the present experiments. Experiments with higher forces are required.

Interpretation of biochemical assays of ADP and Pi release and the weak-to-strong transition have assumed that force generation coincides with the release of Pi (7). This assumption is based on crystal structure experiments that examined the linkage between rotation of the myosin lever arm and occupancy of the active site with various ATP-analogs (17,18). Force generation and relative sliding of myosin and actin can only be measured by mechanical experiments. In fact, optical trap studies of the dwell times of single myosin-Va molecules have also suggested a slow and delayed ADP-release (4,5), consistent with the present experiments. Our report is the first study to directly reveal the relationship between ADP-release and force generation or filament sliding in myosin V.

In a previous study using nonprocessive myosin-II, our group observed ADP release before force generation (11). The Cy3-ATP turnover rate of myosin-II without actin activation was 0.14–0.2 s^{-1} (12,19) and the interval between mechanical displacements was ~8 s (11). Therefore, more than half of the myosins released ADP before rebinding to actin filament. Yet myosin-II still generated a force. This observation suggested that myosin II stored the energy in a protein conformation between ADP dissociation and actin binding. Cy3-ATP turnover rate of myosin-V without actin activation was 0.02 s^{-1} (see Fig. S3 b), which is 10 times longer than that of myosin-II, and the interval between displacements in this study was ~0.6 s (data not shown). In this condition, only ~1% of ADP release occurs before myosin-V rebinds to the actin filament, and most of the mechanical events are directly coupled to ATP turnover. Differences in ADP affinity between myosin types are likely to explain differences in the relationship between ADP release and force generation.

In summary, this study provides direct evidence of slow ADP-release after force generation in myosin V. Because many other processive motors are thought to function like myosin-V (20), these results are likely to have broader implications. Similar results were not observed in the nonprocessive motor myosin-II (11), in which multiple myosin molecules assemble to form a filament and move actin-
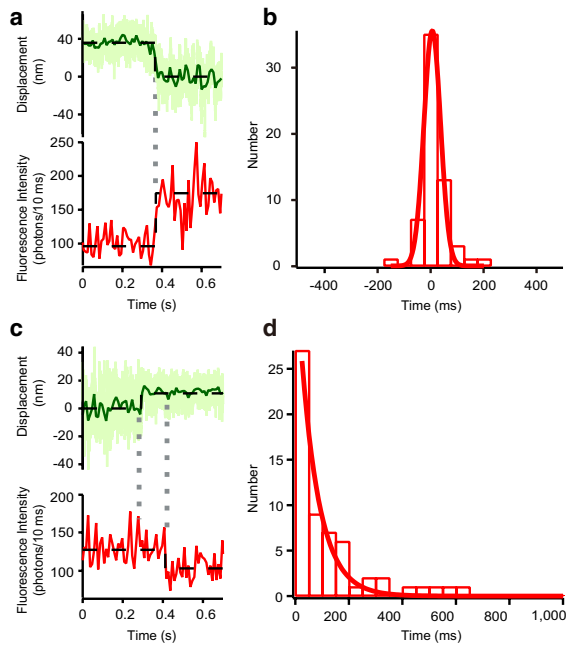
FIGURE 2 Chemo-mechanical coupling in myosin-V. (*a*) The time course of myosin-V detachment (*upper trace*) and Cy3-ATP-binding (*bottom trace*). (*b*) A histogram of the time delay between detachment of myosin-V and binding of Cy3-ATP. Positive time indicates ATP-binding before detachment. The solid line indicates a Gaussian function fit. (*c*) Cy3-ADP-release occurred with a delay after force generation. (*d*) Histogram showing the delay of Cy3-ADP-release following force generation. Two outliers over 1 s were omitted. The solid line indicates a single exponential function fit.

filaments together. In such an assembled system, slow ADP release from a myosin molecule could potentially interfere with the motion generated by other molecules and disrupt motility. Thus, ADP release is likely to be tuned to specialized functions within the myosin family.

SUPPLEMENTARY MATERIAL

Four figures, one table, and additional text are available at www.biophys.org/biophysj/supplemental/S0006-3495(08)00042-8.

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