Supporting Text

α-Actinin-Coated Beads. Ten microliters of 2% (wt/vol) polystyrene latex carboxylated beads (0.945 μm in diameter, Polysciences) was added to 190 μl of 50 mM Mes (pH 5.0). A total of 100 μl of 20 mg/ml EDC (Pierce) in 50 mM Mes (pH 5.0) was added to the solution and incubated for 5 min at 25°C. Then, 300 μl of 450 μg/ml α-actinin in 0.1 M Hepes (pH 8.0) was added and the suspension was incubated for 30 min at 25°C. After the addition of 30 μl of 50 mg/ml BSA, the solution was cooled from 25°C to 4°C. To remove free α-actinin, the beads were rinsed three times in 1 ml of 0.6 M KCl buffer (0.6 M KCl/5 mM $MgCl_2$/1 mM EGTA/20 mM Hepes, pH 7.8) and were then rinsed two times in 25 mM KCl buffer (25 mM KCl/5 mM $MgCl_2$/1 mM EGTA/20 mM Hepes, pH 7.8) by centrifugations. The α-actinin-coated beads were resuspended in 100 μl of 25 mM KCl buffer.

Observation of Fluorescent Cy3-AMP-PNP Bound to Myosin Heads. Cy3-AMP-PNP was synthesized and purified with the same method used for Cy3-ATP as described (1). Fluorescent dye Cy3 was coupled to the ribose ring of AMP-PNP, instead of ATP. More than 99% purity of Cy3-AMP-PNP was acquired by using an HPLC anion-exchange chromatography of Mono Q (Amersham Biosciences, Piscataway, NJ). A solution containing cofilaments was applied to a flow chamber sandwiched between a coverslip and a quartz glass slide with a 5-μm gap. After 3 min, unbound cofilaments were washed out with a 120 mM KCl buffer (120 mM KCl/5 mM $MgCl_2$/1 mM EGTA/20 mM Hepes, pH 7.8). To prevent Cy3-AMP-PNP from nonspecifically binding to the glass surface, the glass surface was coated with 0.1 mg/ml casein (Nacalai Tesque, Kyoto). Then, 100–1,000 nM Cy3-AMP-PNP was added to the chamber for 20 min. Unbound Cy3-AMP-PNP was subsequently washed out with a 120 mM KCl buffer, including an oxygen scavenger system (0.11 mg/ml glucose oxidase/18 μg/ml catalase/2.3 mg/ml glucose/1% 2-mercaptoethanol) to reduce photobleaching (13). Fluorescence from Cy3-AMP-PNP bound to myosin heads in cofilaments was observed within 10 min after washing the cofilaments.

Orientation Dependence of Multiple Successive Large Steps. An M5SH in a cofilament generated successive and nonsuccessive steps (Fig. 3 *a–c* in the main text). The fraction of successive steps (number ratio of successive steps/all observed steps) depended on the angle between the actin filament and the cofilament. The fraction was maximum at ≈70° and almost 0 at <45° and >110°. Thus, M5SH developed successive steps at limited angles (≈70°) and showed nonsuccessive steps at other angles (Table 1 in the main text). It is likely that the angle for myosin heads to continuously move along an actin filament successfully is limited. To examine this point, we measured the rotational diffusion of short (≈2 μm) actin filaments attached to a single M5SH head in cofilaments. Actin filaments thermally rotated over 360° in the absence of nucleotide, indicating high rotational flexibility of the joint between actin filaments and the myosin head on the backbone of a cofilament. However, the actin filaments did not smoothly rotate, but remained stably at ≈70° against the axis of the cofilament for a long time (Fig. 5 *b* and *c*). Interestingly, the stable ≈70° angle coincided with the angle at which the successive steps

of M5SH were observed. This result suggests that whereas the myosin head could interact with an actin filament and develop the first step at any angle, for the myosin head to proceed to successive steps, it is important that the myosin head faces the binding site on an actin filament at ≈70°, which is a favorable angle for rotational diffusion.

The fraction of successive steps of M5DH was weakly but significantly dependent of the angle of actin filaments relative to cofilaments. Interestingly, the favorable angle for successive steps of M5DH (≈45°) was different from that of M5SH. This difference of the favorable angles for successive steps further supports the notion that the successive steps shown in Fig. 3 *a–c* in the main text were indeed due to M5SH but not M5DH.

1. Tokunaga, M., Kitamura, K., Saito, K., Iwane, A. H. & Yanagida, T. (1997) *Biochem. Biophys. Res. Commun.* **235,** 47-53.