Selective Perturbation of the Myosin Recovery Stroke by Point Mutations at the Base of the Lever Arm Affects ATP Hydrolysis and Phosphate Release

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After ATP binding the myosin head undergoes a large structural rearrangement called the recovery stroke. This transition brings catalytic residues into place to enable ATP hydrolysis, and at the same time it causes a swing of the myosin lever arm into a primed state, which is a prerequisite for the power stroke. By introducing point mutations into a subdomain interface at the base of the myosin lever arm at positions Lys84 and Arg704 we caused modulatory changes in the equilibrium constant of the recovery stroke, which we could accurately resolve using the fluorescence signal of single tryptophan Dictyostelium myosin II constructs. Our results shed light on a novel role of the recovery stroke: fine-tuning of this reversible equilibrium influences the functional properties of myosin through controlling the effective rates of ATP hydrolysis and phosphate release.

Various steps of the myosin mechanochemical cycle are linked to large conformational changes of the motor domain, which contains the actin and ATP binding sites as well as the converter region that forms the base of the extended lever arm domain. The converter/lever arm module is thought to amplify protein structural and/or dynamic differences between the ATPase active site to produce a large working stroke (1, 2).

Upon interacting with ATP, the motor domain undergoes a crystallographically identified large structural rearrangement before hydrolysis takes place. During this transition, the movement of the switch-2 loop of the active site toward the γ-phosphate of ATP brings catalytically important residues to their active positions. This open-closed transition of switch-2 is coupled to a large rotation of the lever arm (from down to a primed up state), and thus the conformational rearrangement has been termed the recovery stroke, which constitutes the priming of the myosin head in an actin-detached state. Rebinding of myosin to actin in the post-recovery (up) conformation is a prelude to the power stroke and force generation (3).

Kinetic studies on a Dictyostelium myosin II motor domain construct containing a single ATP-sensitive tryptophan sensor (W501 located in the relay-converter module) have revealed the correspondence between identified structural states and ATPase intermediates (4, 5). In the model shown in Reaction 1, ATP binding to apo-myosin (Mapo3) is followed by the recovery stroke (K3a), which is reversible and rapid compared with the subsequent hydrolysis step (K3b). Following hydrolysis, the reversal of the recovery stroke (K3) is thought to occur before the actual release of Pi (K2). Reaction 1 and the current study deals with the myosin ATPase in the absence of actin, where lever arm motions are uncoupled from the performance of external work. ADP release (K4K5) occurs practically as a reversal of the ATP binding process. (Reaction 1 ignores possible protein structural and/or dynamic differences between the Mdown-ATP, Mdown-ADP, and Mdown-ADP-Pi states.)

\[
\begin{align*}
M^{apo} &\rightarrow M^{apo}-ATP &\rightarrow M^{apo}.ATP &\rightarrow M^{apo}.ATP_{K2} \\
M^{apo}.ATP &\rightarrow M^{down}.ATP &\rightarrow M^{down}.ATP_{K3a} &\rightarrow M^{down}.ATP_{K3b} \\
M^{down}.ATP &\rightarrow M^{down}.ADP &\rightarrow M^{down}.ADP_{K3} &\rightarrow M^{down}.ADP_{K3b} \\
M^{down}.ADP &\rightarrow M^{down}.ADP-Pi &\rightarrow M^{down}.ADP-Pi_{K4} &\rightarrow M^{down}.ADP-Pi_{K4b}
\end{align*}
\]

The above mechanism gives rise to the hypothesis that a shift in the equilibrium constant of the recovery stroke (K3a) will have an effect on the apparent rate constant of ATP hydrolysis (because \( k_{H} \) = \( k_{3b}K_{3a} / (1 + K_{3a}) + k_{-3b} \)). Furthermore, because Pi release is thought to occur in the switch-2 open (down) conformation, the reversal of the recovery stroke (K3) may influence the steady-state ATPase rate, which is controlled by the effective rate of Pi release. Thus, besides its role in ATP hydrolysis and priming the myosin head, the recovery stroke may be important in fine-tuning the steady-state distribution of myosin molecules in up and down lever arm orientations and, in

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3 The abbreviation used is: M, myosin; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid.
turn, the strong and weak actin binding states during the contractile cycle.

To test this hypothesis, we designed Dictyostelium myosin II motor domain mutants to perturb the recovery stroke selectively. This step is accompanied by a large rotation of the converter region that causes disruption of its interface to the N-terminal subdomain of the myosin head (Fig. 1, A and B). One good candidate for a directed change in the interaction pattern of this interface is the Lys\textsuperscript{84} (N-terminal subdomain)-Arg\textsuperscript{704} (converter) residue pair. In the down (pre-recovery) conformation the two side chains, unusually, run almost parallel to each other and their positive charges are separated by only \(~0.5\) nm (Fig. 1A). In the up (post-recovery) structure this complex is disrupted (Fig. 1B), implying that the introduction of site-directed substitutions in these positions will possibly affect the recovery stroke equilibrium constant. We constructed and characterized the K84M point mutant in which the positive charge of Lys\textsuperscript{84} was removed and the side chain replaced by a roughly isosteric one and R704E in which we intended to convert the originally repulsive Lys\textsuperscript{84}-Arg\textsuperscript{704} interaction into a salt bridge. We introduced these point mutations into the W501\textsuperscript{1} construct, which has been shown to enable very sensitive resolution of the recovery stroke, while retaining essentially identical kinetic properties to the wild-type enzyme (4, 5). In later sections we will refer to the W501\textsuperscript{+} control as “wild-type” for simplicity.

**EXPERIMENTAL PROCEDURES**

*Protein Expression and Purification*—All constructs used in this study are derivatives of the M761 Dictyostelium myosin II motor domain (6). Construction of the plasmid for the W501\textsuperscript{+} motor domain containing the point mutations W36F, W432F, and W584F was described earlier (4). The W501\textsuperscript{+} construct was further mutagenized to yield the K84M and R704E mutants as described previously (7). The pDXA-3H expression vector was used for constitutive expression of the motor domains (8). Dictyostelium AX3 cells were transformed and cultured and the recombinant proteins were prepared as described previously (4). Protein concentrations were determined using Bradford reagent (Sigma). Purity of the preparations (>95%) was checked by 9% SDS-PAGE.

*Nucleotides and Nucleotide Analog Complexes*—ATP (special quality, vanadate-free) was from Roche Applied Science. Other nucleotides were purchased from Sigma. The

![Figure 1](image-url)
M:ADP-BeF₃ complex was prepared by incubation of 5–20 μM motor domain, 50 μM ADP, 3 mM NaF, 50 μM BeCl₂ for 30 min. The M:ADP-AlF₃ complex was made similarly but with 50 μM AlCl₃ instead of BeCl₂ and incubation for at least 2 h.

**Kinetic and Spectroscopic Measurements**—All measurements were carried out in a buffer comprising 20 mM TES, pH 7.5, 40 mM NaCl, and 2 mM MgCl₂ as described previously (4, 5, 9). Reaction profiles were analyzed by fitting to exponential functions using Origin v7.5 (Microcal Software).

**Molecular Dynamics Simulations**—Protein Data Bank structure 1FMW and its single point mutants were used as inputs in the modeling studies. Energy minimization and subsequent 5-ns molecular dynamics equilibration were performed with the GROMACS program package (10). 17800 explicit water molecules surrounded the proteins during calculations.

**RESULTS**

**Structural Integrity of the Mutants**—Molecular dynamics simulations of the wild-type and mutant proteins in a water box showed that the amino acid substitutions did not cause significant structural changes even in the vicinity of the targeted side chains and that the structures were stable over a 5-ns time scale (Fig. 1, C–E). The charge separation between Lys⁸⁴ and Glu⁷⁰⁴ in the R704E mutant was rather similar to that between Lys⁸⁴ and Arg⁷⁰⁴ of the wild-type enzyme (0.5 nm). Also, the side chain of Met⁸⁴ in the K84M mutant adopted a very similar conformation to the corresponding Lys⁸⁴ of the wild-type construct and had a similar proximity to Arg⁷⁰⁴.

**Steady-state ATPase Activities**—The basal (actin-free) steady-state ATPase activity of K84M was ~1.5 times, and that of R704E was ~3 times, higher than that of the wild-type construct (Table 1). In addition to the NADH-linked assay, ATPase activities were confirmed by multiple turnover tryptophan fluorescence emission spectra of the studied constructs (Table 1). In addition to the NADH-linked assay, ATPase activities were confirmed by multiple turnover tryptophan fluorescence emission spectra of the studied constructs (Table 1). In addition to the NADH-linked assay, ATPase activities were confirmed by multiple turnover tryptophan fluorescence emission spectra of the studied constructs (Table 1). In addition to the NADH-linked assay, ATPase activities were confirmed by multiple turnover tryptophan fluorescence emission spectra of the studied constructs (Table 1).

**Tryptophan Fluorescence Changes on Nucleotide Interaction**—In our earlier studies, W501 showed a small quench on ADP binding (transition from apo to down conformation), whereas ATP binding caused a large fluorescence increase as the up state became populated (4, 5, 11) (Reaction 1). Fig. 2 shows tryptophan fluorescence emission spectra of the studied constructs in the absence of nucleotide, and in ADP and ATP. The extent of the quench caused by ADP was similar in all constructs, whereas the fluorescence increase on ATP addition was markedly smaller in R704E than in the other two constructs. The blue shifts characteristic of both ADP and ATP binding to apo-W501 + were not affected by the mutations (Fig. 2).

We measured the temperature dependence of the tryptophan fluorescence emission of the studied constructs (Fig. 3, A–C). The slope of the temperature dependence of the wild-type construct in the absence of nucleotide, in ADP, and ADP-AlF₃ (an ADP-P₃ analog) was similar to that of free tryptophan, implying that the motor domain adopts three distinct conformations with different fluorescence intensities in these cases (apo in the absence of nucleotide, down in ADP, and up in ADP-AlF₃) (Fig. 3A) (5). The temperature dependence was clearly different in ATP and in the ATP analogs ADP-BeF₃ and AMPPNP. The intermediate intensities and markedly different slopes in these cases have been interpreted by means of the formation of a temperature-dependent reversible equilibrium of the down and up states, i.e., the recovery stroke (Fig. 3A) (5).

In the apo, ADP (down), and ADP-AlF₃ (up) states, the K84M and R704E constructs showed similar profiles to those of the wild type, indicating that the gross structures of these states are unchanged by the mutations (Fig. 3, A–C). For ADP-BeF₃ and AMPPNP, the equilibrium constant of the recovery stroke (Kₐ₋₃) could be calculated from fluorescence intensities as Kₐ₋₃ = (Fₓ/Fₐ₋₃) (Fₐ₋₃/Fₓ) where Fₓ and Fₐ₋₃ are the fluorescence intensities in the down (ADP-bound) and up (ADP-AlF₃-bound) states, respectively, and Fₐ₋₃ is the intensity of the given nucleotide analog complex (Fig. 3, A–C).

**Molecular Dynamics Simulations**—Protein Data Bank structure 1FMW and its single point mutants were used as inputs in the modeling studies. Energy minimization and subsequent 5-ns molecular dynamics equilibration were performed with the GROMACS program package (10). 17800 explicit water molecules surrounded the proteins during calculations.

**TABLE 1**

| Kinetic and thermodynamic properties of motor domain constructs |
|---------------------------------------------------------------|
| Rate and equilibrium constants are based on Reaction 1.        |
|                                                               |
| **T** | Wild-type* | K84M | R704E |
|-------|------------|------|-------|
|       | °C         |      |       |
| Steady-state basal ATPase activity (s⁻¹)                        |
| ATP binding and hydrolysis                                     |
| Kₑ (μM⁻¹ s⁻¹)                                                  |
| apo            | 20 0.05 ± 0.01 | 0.08 ± 0.01 | 0.17 ± 0.03 |
| Kₑ (μM⁻¹ s⁻¹) apo |
| 5 3.4 ± 0.1 | 2.0 ± 0.03 | 1.8 ± 0.01 |
| 30 53 ± 0.1 | 56 ± 0.03 | 73 ± 0.03 |
| Recovery stroke                                              |
| AMPPNP                                                     |
| Kₑ (μM⁻¹ s⁻¹) apo |
| 20 0.27 ± 0.02 | 0.20 ± 0.02 | 0.11 ± 0.04 |
| Kₑ (μM⁻¹ s⁻¹) apo |
| 25 1.4 ± 0.3 | 0.80 ± 0.01 | 0.40 ± 0.04 |
| ADP binding and release                                       |
| Kₑ (μM⁻¹ s⁻¹) apo |
| 20 1.8 | 0.82 | 1.5 |
| 40 110 ± 160 | 100 ± 100 | 100 ± 100 |
| 60 590 ± 100 | 300 ± 20 | 200 ± 20 |
| Kₑ (μM⁻¹ s⁻¹) apo |
| 20 5,9 | 8,8 | 5,2 |
| 20 110 | 42 | 110 |
| 110 |
| 9,2 | 8,8 | 5,3 |

* W501 construct (see Introduction).
* Apparent rate constant of the ATP hydrolysis step (see "Results").
* Calculated from tryptophan fluorescence intensities as Kₑ = (Fₓ/Fₐ₋₃) (Fₐ₋₃/Fₓ) where Fₓ and Fₐ₋₃ are the fluorescence intensities in the down (ADP-bound) and up (ADP-AlF₃-bound) states, respectively, and Fₐ₋₃ is the intensity of the given nucleotide analog complex (Fig. 3, A–C).
* Calculated from the van’t Hoff plots of the recovery stroke (ln Kₑ = -ΔHₑ/RT + ΔSₑ/R). (Fig. 3, D and E).
* Calculated from the hyperbolic dependence of the kₑo of ADP binding transients on [ADP] (kₑo = kₑ [ADP][Kₐ₋₃] + kₑ) (Fig. 6A).
* From ADP shaking experiments (Fig. 6B).
* Equilibrium dissociation constant of ADP binding (Kₑ = Kₑo).

In the apo, ADP (down), and ADP-AlF₃ (up) states, the K84M and R704E constructs showed similar profiles to those of the wild type, indicating that the gross structures of these states are unchanged by the mutations (Fig. 3, A–C). For ADP-BeF₃ and AMPPNP, the equilibrium constant of the recovery stroke (Kₐ₋₃) could be calculated from fluorescence intensities as Kₐ₋₃ = (Fₓ – Fₐ₋₃) (Fₐ₋₃ – Fₓ) where Fₓ and Fₐ₋₃ are fluorescence intensities in the presence of the given nucleotide, ADP (down state), and ADP-AlF₃ (up state), respectively. The equilibrium parameters showed that the K84M mutation, and even more so the R704E mutation, caused marked shifts in favor of the down conformation (Table 1). In ATP the situation is more complex because the apparent equilibrium constant of the recovery stroke (Kₑo/Kₑ) is also affected by the hydrolysis step (Kₑo/Kₑ = Kₐ₋₃ (1 + Kₐ₋₃), Reaction 1), but a shift toward the down state in R704E is evident in this case (Fig. 3, A–C).

Although the mutations shifted the equilibrium constant of the recovery stroke (Kₐ₋₃) several times, these have only small perturbations in the overall energetic profile of this step. The small ΔGₑ associated with the recovery stroke is composed of a large enthalpy increase (ΔHₑ = 50–100 kJ/mol) and a compensating entropic contribution (Fig. 3, D and E, Table 1) in all mutants and the wild type.
Kinetics of ATP Interaction—On mixing the W501 motor domain with ATP in the stopped-flow, the two-step ATP binding process ($K_1 k_2$ in Reaction 1) is followed by the more rapid recovery stroke ($K_3a$) and the subsequent hydrolysis step ($K_3b$) (4, 5). The kinetics of the initial phase of the tryptophan fluorescence transients is dictated by nucleotide binding ($K_1 k_2$), but the amplitude of this phase will be set by the equilibrium constant of the oncoming rapid recovery stroke ($K_3a$). Because the W501 fluorescence intensity in the down state is lower and in the up state is higher than that in the apo state, the initial phase can be either a burst (fluorescence increase) or a quench (fluorescence drop), depending on the value of $K_3a$. After this initial phase, there is a subsequent fluorescence enhancement indicating that the hydrolysis step ($K_{3b} \gg 1$) pulls the reaction over to result in the predominance of the high fluorescence Mup/ADP/Pi state (Reaction 1). The observed rate constant ($k_{obs}$) of this second phase (i.e. the apparent rate constant of ATP hydrolysis, $k_{appH}$) will be a composite of the recovery stroke equilibrium constant and the rate constants of the hydrolysis step ($k_{appH} = k_{3b} K_3a / (K_{3a} + 1) + k_{-3b}$; Reaction 1) (4, 5).

The dependence of the $k_{obs}$ of the initial phase on ATP concentration was quasilinear in all mutants, showing signs of saturation above 1 mM ATP ($K_1 \leq 10^{-3} \mu M^{-1}$, data not shown). The plots had slopes ($K_1 k_2$) ~ 0.5 $\mu M^{-1} s^{-1}$ at 5°C, and ~1 $\mu M^{-1} s^{-1}$ at 20°C, consistent with earlier wild-type data and showing little effect of the mutations on ATP binding kinetics (Table 1) (4). However, a more pronounced initial quench (or a lack of an initial burst) together with a slower second phase (smaller $k_{appH}$) in the mutants compared with the wild-type demonstrated a marked reduction in the recovery stroke equilibrium constant ($K_{3a}$) caused by the mutations (Fig. 4, A and B). Fig. 4C shows the $k_{obs}$ of the second phase at 20°C plotted against ATP concentration. A marked reduction in the maxi-
mal k_{obs} was observed in K84M and even more in R704E. This tendency held throughout the temperature range studied (5–20 °C, Fig. 4D). Analysis of the amplitude data of the stopped-flow transients also highlighted the correspondence between the recovery stroke equilibrium constant (K_{3a}) and the apparent rate constant of the hydrolysis step (app k_{H}). The fluorescence levels after the initial phase were lower in K84M and even more in R704E than in the wild-type (indicating a smaller K_{3a}), whereas in the second phase ATP hydrolysis (K_{3b}) pulled the reaction to the high fluorescence M^{AP-ADP-P}_f state in all cases (Fig. 4E, Reaction 1).

Kinetics of the Recovery Stroke—Because the recovery stroke is much faster than both the preceding and subsequent steps, its kinetics can be investigated only by its selective perturbation using relaxation kinetic techniques (5, 12). Following a rapid pressure-jump, the motor domain-nucleotide analog (ADP{BeFx and AMPPNP) complexes showed conformational relaxation of the reversible and pressure-sensitive recovery stroke (Fig. 5A). The k_{obs} values of these tryptophan fluorescence transients is the sum of the forward and reverse rate constants (k_{obs} = k_{3a} + k_{-3a}). k_{3a} and k_{-3a} in the ADP-BeFx and AMPPNP complexes of the motor domain could thus be determined from the k_{obs} of the pressure-jump records and the K_{3a} equilibrium constants determined in equilibrium fluorescence measurements (Fig. 3, Table 1).

Fig. 5B shows that the shift toward the down state (a reduction in K_{3a}) caused by the mutations is brought about predominantly by a reduction in k_{3a}, whereas the reverse rate
constant ($k_{-3a}$) remains largely unaffected. Furthermore, in the Arrhenius plots of these rate constants the slopes of ln $k_{3a}$ or ln $k_{-3a}$ versus 1/T were very similar in all constructs, whereas the $k_{3a}$ values of the mutants were markedly offset from those in the wild-type (Fig. 5, C and D). This means that the mutations caused a reduction in the pre-exponential term of the Arrhenius equation, but the exponential term remained largely unchanged ($k = A \exp(E^*/RT)$ where A is the pre-exponential term and $E^*$ is the activation energy; Table 2).

Kinetics of ADP Binding and Release—On mixing with excess ADP in the stopped-flow, all constructs showed a single exponential quench in tryptophan fluorescence (traces not shown). Analysis of the hyperbolic dependence of $k_{obs}$ on ADP concentration ($k_{obs} = k_{-6i}([ADP])/(K_7 + [ADP]) + k_6$) showed that the initial low affinity binding step ($K_7$) was not affected by the mutations, whereas the rate constant of the subsequent isomerization ($k_{-6i}$) was slightly reduced (Fig. 6A, Table 1, Reaction 1).

The rate constants of ADP dissociation ($k_6$) were determined by chasing experiments in which the motor domain-ADP complexes were mixed with high concentrations of ATP in the stopped-flow (Fig. 6B). The mutations had no profound effect on either $k_6$ or the overall ADP binding equilibrium constant ($K_{eq} = k_5/k_6$; Table 2).

DISCUSSION

Selective Perturbation of the Recovery Stroke by Subdomain Interactions—We aimed to selectively perturb the myosin recovery stroke by converting the repulsive Lys$^{84}$-Arg$^{704}$ interaction into a weak attraction in the K84M mutant and a salt bridge in the R704E construct (Fig. 1). These mutations are located at the N-terminal subdomain-converter interface, which is far from the nucleotide binding pocket. As judged from the kinetics of nucleotide binding to and release from the mutants as well as molecular dynamic simulations, the substitutions did not affect the overall structure of the nucleotide binding site (Fig. 1). However, the mutations had a distinct effect on the recovery stroke that involves marked conformational rearrangements involving distant parts of the molecule. Thus, the studied mutations modulated surface charges sufficiently to have an effect on recovery stroke kinetics, but they did not override the structures of the start and end points of this transition. In all constructs ADP induced the same pre-recovery (switch-2 open or lever arm down) state and ADP-AIF$_4$ induced the post-recovery (switch-2 closed or lever arm up) state, as judged by W501 emission intensity (Fig. 3, A–C). Other attractive forces across the subdomain interface in the wild type must dominate over the repulsive force between Lys$^{84}$ and Arg$^{704}$ in the down state in order to bring them to within 0.5 nm. The wild-type Lys$^{84}$-Arg$^{704}$ repulsive interaction exists only in the down state, and we show that this state becomes more favorable in the order wild-type → K84M → R704E.

It should be noted that the R704E mutation would also disrupt a salt bridge to Glu$^{755}$ within the same subdomain; however, this appears to have little consequence on the overall properties of this construct. In particular, in the K84M mutant where the Arg$^{704}$-Glu$^{755}$ salt bridge was intact, the kinetic and thermodynamic effects on the recovery stroke were perturbed in the same direction as in the R704E construct.

The Recovery Stroke Is Coupled to ATP Hydrolysis and P$_i$ Release—By characterizing the transient fluorescence profiles of the interaction of the mutants with ATP and their steady-state ATPase activity, we demonstrate that even a moderate decrease in the equilibrium constant of the rapid and reversible recovery stroke causes significant slowing of ATP hydrolysis, which takes place after the recovery stroke (Fig. 4, Table 1, Reaction 1). Conversely, P$_i$ release, which is the rate-limiting step of the basin ATPase cycle and takes place after the reversal of the recovery stroke ($K_4$), is accelerated by the same mutations (Table 1, Reaction 1). This finding is consistent with earlier work in which trinitrophenyl-
Fine Tuning of the Myosin Recovery Stroke

loration of Lys84 caused a marked increase in the basal steady-state ATPase activity of Dictyostelium myosin II because the pre-recovery state became predominant during the enzymatic cycle (13).

Mechanism of the Recovery Stroke—We show that the studied mutations selectively decreased the forward rate constant of the recovery stroke ($k_{3a}$), whereas they did not have a remarkable effect on the reverse rate constant ($k_{3b}$) (Fig. 5B, Table 2). The structural basis of the asymmetric effect is the disruption of the subdomain interface containing residues 84 and 704 upon the recovery stroke that moves these residues ~3.8 nm apart from each other (Fig. 1, A and B) (1). Because only a short-range interaction can be formed between these side chains, the mutations do not affect the reverse rate constant ($k_{3b}$).

It is surprising that, although the mutations targeted a specific side chain interaction that could be expected to have an enthalpic character, the pre-exponential factor changed rather than the apparent activation energy of the recovery stroke (Fig. 5, C and D, Table 2). The mutations investigated in the present study affect short-range interactions in the pre-recovery conformation, and therefore they limit the entry into the pathway leading to the conformational change and possibly reshape the diffusion landscape in the initial part of the pathway. The recent simulations of the reaction coordinate of the myosin recovery stroke using Conjugate Peak Refinement provide some indications as to the nature of this pathway (14). Protein conformational changes that gate enzyme activity and protein folding are likely to share these characteristics.

Role of the Recovery Stroke Equilibrium in the Myosin Mechanism—By affecting the effective rate of ATP hydrolysis and $P_i$ release, the equilibrium constant of the recovery stroke may influence the distribution of steady-state enzymatic intermediates during the functioning of myosins. Given the widely varying actin affinities of these intermediates, this effect may also influence the duty ratio, i.e. the fractional occupancy of strongly actin-bound myosin states during steady-state ATPase cycling. The duty ratio is a key functional parameter for the effective functioning of different motors. (For instance, ensemble motors such as muscle myosin II exhibit a low duty ratio, whereas single-molecule stepping by myosin V requires a high duty ratio.) Indeed, the ATP hydrolysis step is quite reversible in most myosins examined: the apparent equilibrium constant of the hydrolysis of enzyme-bound ATP ($^{app}K_{3a}$) is between 0.3 and 9 for various myosins (15–20). Because the chemical step is coupled to the recovery stroke (5, 12), the recovery stroke equi-