ADP Binding Induces an Asymmetry between the Heads of Unphosphorylated Myosin*

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Light chain phosphorylation is the key event that regulates smooth and non-muscle myosin II ATPase activity. Here we show that both heads of smooth muscle heavy meromyosin (HMM) bind tightly to actin in the absence of nucleotide, irrespective of the state of light chain phosphorylation. In striking contrast, only one of the two heads of unphosphorylated HMM binds to actin in the presence of ADP, and the heads have different affinities for ADP. This asymmetry suggests that phosphorylation alters the mechanical coupling between the heads of HMM. A model that incorporates strain between the two heads is proposed to explain the data, which have implications for how one head of a motor protein can gate the response of the other.

A common feature of the myosin II family is that all of its members contain two heads connected to an α-helical coiled-coil tail. Although the single-headed subfragment of myosin (S1) has always been considered a good model system for studying myosin structure and function, it is clear that some important features of whole myosin may be overlooked by studying only this model species. A prominent example is that phosphorylation-dependent regulation of actin-activated ATPase activity of smooth and vertebrate non-muscle myosin II is manifested only in subfragments that have two heads as well as a minimal length of the coiled-coil tail (1). In contrast, single-headed myosin is unregulated and always active (2). Tethering of the two heads together via the coiled-coil tail can also impose steric and mechanical constraints on the interaction of the two heads with actin. This has been explored in the case of skeletal muscle heavy meromyosin (3, 4) (HMM) but not for the regulated HMMs, where light chain phosphorylation may also be expected to affect the coupling between the two heads.

Structural differences between phosphorylated and unphosphorylated HMM in the presence of ATP were recently revealed through analysis of two-dimensional crystalline arrays by image reconstruction and docking of high resolution structures into the observed density maps (5). In the phosphorylated state the two heads showed no contact with each other. In contrast, the unphosphorylated HMM showed an interaction between the two heads, with the actin-binding interface of one head bound to the converter domain of the other. This asymmetrical interaction between the unphosphorylated heads suggested a mechanism for how phosphorylation-dependent regulation of activity is achieved. One head cannot interact with actin and therefore its ATPase activity will not be enhanced, whereas the other head cannot undergo the rotation of the converter domain that is necessary to achieve phosphate release (5).

This unexpected structure provided evidence that the coupling between the heads of double-headed smooth muscle heavy meromyosin is influenced by the state of light chain phosphorylation. To further understand how the two heads of smooth muscle HMM interact with each other, we undertook a kinetic analysis of HMM in both the unphosphorylated and phosphorylated states. In addition to the kinetic changes that account for regulation of ATPase activity, another major difference was detected in the strong binding states of the cross-bridge cycle. Although both heads of phosphorylated HMM bind to actin in the presence of ADP, only one head of unphosphorylated HMM binds to actin under these conditions. This observation not only shows that light chain phosphorylation changes the mechanical coupling between heads but also provides a mechanism whereby ADP binding to one head can gate the response of the second head.

EXPERIMENTAL PROCEDURES

Protein Preparation—Recombinant baculovirus was isolated by conventional protocols (7). For HMM expression, Sf9 cells in suspension culture were coinfected with two recombinant viral stocks, one coding for the heavy chain (amino acids 1–1175) and one coding for the regulatory light chain and essential light chains. The heavy chain was cloned with a FLAG tag at the C terminus to facilitate purification (DYKDDDDK). The cells were harvested at 65–75 h, and the recombinant proteins were isolated on an anti-FLAG affinity column (Sigma). Unlike proteolytically prepared HMM, the expressed HMM yielded a homogeneous product with intact heavy and light chains. Thio phosphorylated HMM was prepared by incubation of unphosphorylated HMM with ATP–γ-S, CaCl₂, myosin light chain kinase, and calmodulin. The protein was flash frozen in liquid nitrogen in the presence of 3 mg sucrose/mg protein and stored at −80 °C. F-actin was prepared following the method of Spudich and Watts (8) and labeled with pyrene as described previously (9).

Transient Kinetics—All experiments were carried out at 20 °C in 20 mM MOPS, 5 mM MgCl₂, and 100 mM KCl, pH 7, unless specified. Kinetics measurements used a standard Hi-Tech SF-61 DX2 stopped flow spectrophotometer using a 100W Xe/Hg lamp and a monochromator for wavelength selection. Pyr-actin and mantATP fluorescence was excited at 365 nm, and emission was detected after passing through a
389-nm cutoff filter. Tryptophan fluorescence was excited at 295 nm, and the emission was detected after passing through a 320-nm cutoff filter. All of the transients shown are the average of three to six shots of the stopped flow apparatus superimposed with the best fit of a single or double exponential function. In kinetic experiments it is normal to quote the concentrations of the reacting species after mixing in the stopped flow (dilution by 2, 1:1 mixing). Where the amplitudes are analyzed to provide information on the concentration of a species present at equilibrium, then the concentration before mixing is relevant. In Figures, the concentrations are quoted as initial or final as appropriate.

RESULTS

ATP and ADP Induced Dissociation of Acto-HMM—When a myosin head binds to an actin molecule that has been covalently labeled with pyrene iodoacetamide (pyr-actin) the fluorescence of the pyrene group is quenched by up to 70%. Dissociation of HMM from pyr-actin by ATP was monitored by the increase in fluorescence of the pyrene group (3, 9). On adding excess ATP to pyr-acto-HMM the observed transient was a single exponential with a similar observed rate constant ($k_{obs}$) and amplitude for both thiophosphorylated (thioP) and unphosphorylated (uP) HMM (Fig. 1). The apparent second order rate constant for ATP binding to HMM was $k_{obs} = 42$ s$^{-1}$ in the presence of ATP, and $k_{obs} = 4.01$ s$^{-1}$ with ADP. Buffer conditions are 30 mKCl, 20 mM MOPS, and 5 mM MgCl$_2$. ADP was treated with hexokinase and AP5A as previously described (19, 20).

ADP was added to acto-uP-HMM, a fluorescence increase was observed which was approximately half of the amplitude of that seen for ATP with a $k_{obs}$ of 0.41 s$^{-1}$. This result indicates that half of the unphosphorylated myosin heads are no longer tightly bound to actin. Note that the experiment is done with a 2-fold excess of actin over HMM. Loss of half of these tightly bound heads means that only one in four of the actins has a tightly bound myosin head.

Affinity of HMM for Actin—The affinity of pyr-actin for thioP-HMM and uP-HMM in the absence of nucleotide was measured by incubating HMM with phalloidin-stabilized pyr-actin and then mixing with excess ATP (10). The amplitude of the dissociation reaction was linearly dependent on HMM concentration until a plateau was reached for both thioP- and uP-HMM (Fig. 2, A and B), respectively, in presence and absence of 100 μM ADP. The data are analyzed either as a linear fit or as a titration curve (see “Experimental Procedures”). Buffer conditions are as for Fig. 1 except for ATP where KCl = 200 mM and for ADP where KCl = 30 mM KCl.

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Effect of ADP on Acto-HMM Affinity—The affinity of actin for HMM-ADP was measured by repeating the assay described.

![Fig. 1](image1.png)

**Fig. 1.** Pyrene fluorescence changes on mixing pyr-actin (200 nM) and HMM (100 nM) with 100 μM ATP or 300 μM ADP (initial concentrations). A, thioP-HMM with $k_{obs} = 33$ s$^{-1}$. B, uP-HMM with $k_{obs} = 42$ s$^{-1}$ in the presence of ATP, and $k_{obs} = 4.01$ s$^{-1}$ with ADP. Buffer conditions are 30 mM KCl, 20 mM MOPS, and 5 mM MgCl$_2$. ADP was treated with hexokinase and AP5A as previously described (19, 20).

![Fig. 2](image2.png)

**Fig. 2.** Titration of pyrene-actin with thioP-HMM and uP-HMM in the presence or absence of ADP. 40 μM ATP was mixed with 40 nM phalloidin-stabilized pyr-actin and various concentrations of HMM. The amplitude of the ATP-induced dissociation of pyr-actin complex is plotted against HMM concentration before mixing thioP-HMM (A) and uP-HMM (B), respectively, in presence and absence of 100 μM ADP. The data are analyzed either as a linear fit or as a titration curve (see "Experimental Procedures"). Buffer conditions are as for Fig. 1 except for ATP where KCl = 200 mM and for ADP where KCl = 30 mM KCl.
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Table I
Data from the actin-HMM titrations

| Protein | KCl (mM) | Fm (+ADP) | Fm (+ADP) | Fm (+ADP) |
|---------|----------|-----------|-----------|-----------|
| thioP-HMM | 30 | 0.44 ± 0.08 | 0.35 ± 0.03 | 0.32 ± 0.01 |
| uP-HMM  | 30 | 0.25 ± 0.07 | 0.17 ± 0.01 | 0.13 ± 0.02 |
| S1      | 200 | 0.30 ± 0.05 | 0.25 ± 0.01 | 0.22 ± 0.02 |

Table II
Afinity of ADP for the binding sites on HMM

| Protein  | KADP1 (µM) | KADP2 (µM) |
|----------|-------------|-------------|
| thioP-HMM | 0.7         | 0.7         |
| uP-HMM   | 2           | v. weak     |
| acto-thioP-HMM | 10       |             |
| acto-uP-HMM | 1–2       | 10–20       |

The data is that only one head of uP-HMM can make a rigor-like R-state complex with actin in the presence of ADP. Because the saturation point is independent of ADP, either the second head is binding in the weaker attached or A-state, which does not give a fluorescence signal change, or the second head is sterically preventing the binding of a second HMM to that actin site. Affinity of ADP for Acto-HMM—Because ADP induces a dramatic change in the binding of uP-HMM to actin we determined the affinity of ADP for acto-HMM. The pyr-acto-HMM complex, in the presence of increasing concentrations of ADP, was dissociated by mixing with a large excess of ATP (Fig. 3). The best fit to the sum of one or two exponentials is superimposed on the experimental traces (fast phase ~350 s⁻¹, slow phase ~40 s⁻¹). The fast phase arises from heads without nucleotide that bind ATP very quickly and dissociate from actin. The slow phase results from heads that have ADP at the active site and the rate of ATP binding is limited by the rate of ADP dissociation. A comparison of the amplitudes of the fast and slow components allowed the fraction of HMM heads occupied by ADP to be estimated, and thus the affinity of the head for ADP to be deduced (11).

The total amplitude was independent of ADP concentration as expected if all heads remain tightly bound to actin in the presence of ADP (Fig. 3B). The fast and slow phase amplitudes were plotted as a fraction of the total amplitude at each ADP concentration (Fig. 3B). A fit of the two curves to a hyperbola gave similar binding constants, KADP, of 10.8 µM for the titration of the increase of the slow phase and decrease of the fast phase (Table II). In all of these plots the initial concentration of ADP is plotted because this is the concentration when the ADP binding is at equilibrium just before the addition of ATP.

The same experiment was repeated with uP-HMM. The interpretation of this titration was not as simple as for thioP-HMM.
HMM because the two effects are apparent. The total amplitude decreases as tightly bound heads are lost at increased ADP concentration. At 100 μM the total amplitude has decreased by 50% and little further change is expected based on the results in Figs. 1 and 2. ADP concentrations greater than 100 μM cannot be used because at higher concentrations the fast and slow amplitudes are no longer distinct due to a reduction in the rate of the fast phase because of competition of ATP with ADP. The loss of total amplitude appears monophasic and required 10–20 μM ADP for half of the effect. The change in fractional amplitude of the fast and slow phases showed two components. The transient appears as 50% fast and slow phase at 16 μM ADP with half-saturation at 1–2 μM suggesting an ADP affinity of 1–2 μM for half of the sites. The remainder of the fast phase required much higher concentrations of ADP to eliminate it and was similar to the concentration dependence seen in the total amplitude plot, consistent with an affinity of 10–20 μM. This leads to the conclusion that 50% of the acto-HMM sites bind ADP with an affinity of 1–2 μM, and the remainder bind with a much lower affinity (10–20 μM).

Affinity of ADP for HMM in the Absence of Actin—The binding of excess ATP to HMM results in a ∼10% increase in protein fluorescence that could be fitted by a single exponential at all ATP concentrations. The value of k_{obs} was linearly dependent upon ATP concentration from 0–30 μM with the same slope for both uP-HMM and thioP-HMM (1.2 μM s^{-1}) (12). Because ADP binds HMM with a much smaller fluorescence change than ATP, the displacement of ADP can be monitored from the net increase in fluorescence as ATP replaces ADP. ThioP-HMM was preincubated with various ADP concentrations and then mixed with excess ATP (Fig. 4A). In the presence of ADP the reaction is biphasic. The fast phase occurred at 21 s^{-1}, which is the k_{obs} for ATP binding to any unoccupied thioP-HMM. The slow phase occurred at the k_{obs} for ADP dissociating from thioP-HMM and was 3 s^{-1}. As the ADP concentration is increased, the amplitude of the slow phase increases while that of the fast phase decreases. The best fit to a hyperbola (Fig. 4B) gives a K_{ADP} of 0.7 μM for both phases. The decrease in total amplitude is consistent with the fact that ADP binding to HMM causes a smaller fluorescence enhancement than does ATP binding.

The same experiment was repeated with uP-HMM (Fig. 4, C and D). The observed rate constant of the fast phase was ∼30 s^{-1}, and that of the slow phase was 2.4 s^{-1}. The total amplitude decreases at the same time as the proportion of fast and slow amplitudes changes (Fig. 4D). The total amplitude decreases by half at 40 μM ADP and at the same time the remaining amplitude is 50% fast and slow phase. The best fit to hyperbolae for the slow and fast phase data gave similar values of K_{ADP} of 2 μM. The total amplitude shows the same concentration dependence. The loss of total fluorescence is large and unexpected and remains to be explained. If the two heads are binding ADP differentially, then we have no simple way to assign the amplitudes of the fluorescence changes to particular nucleotide binding states. The observation that half of the observed transient is compatible with ATP binding unhindered to the nucleotide site suggests that approximately half of the sites have no tightly bound ADP. This suggests that half of the sites are binding ADP with an affinity of 2 μM, and the remainder have a much weaker affinity and may not bind ADP at all under these conditions.

The asymmetry in the behavior of uP-HMM shown here does not result from heterogeneity in the protein. The recombinant HMM heavy chain is intact, and the species that were analyzed were either completely phosphorylated or completely unphosphorylated within the limits of detection by gels. Multiple turnover assays in the stopped flow gave ATPases of 0.019 s^{-1} for thioP-HMM and 0.0024 s^{-1} for uP-HMM indicating a good level of regulation. Single turnover assays based on the displacement of a fluorescent ATP analogue from HMM in the steady state by unlabeled ATP (13, 14) showed that 85–90% of the transient was described by a single exponential with a k_{obs} of 0.0008 s^{-1} and a small fraction (10–15%) with a k_{obs} of ∼10 times faster (giving an average turnover rate of 0.0019 s^{-1}). The results were similar using either mantATP or mant-deoxyATP. Thus the 50% heterogeneity of the behavior of uP-HMM cannot be accounted for by simple heterogeneity in the protein preparations.

**DISCUSSION**

One-headed Versus Two-headed Binding—The affinity of HMM for actin in the absence of nucleotide is stronger than S1 as expected from all other studies of dimeric versus monomeric myosin fragments (3, 15). Because of the high affinity, it is not
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FIG. 5. A, The conformational changes of smooth muscle myosin S1 on actin. The first conformational change (shown here as a bend of the S1 around a hinge in the middle of the molecule) is associated with P release and is the power stroke moving the tip of the S1 neck by 5–10 nm. This is followed by a second conformational change associated with ADP release, which moves the neck a further 3–5 nm in the same direction as the power stroke (19, 21). B, if the two heads of HMM bind in the rigor conformation to two adjacent actins, then because the two actins are 5.5 nm apart in the filament there must be 5.5 nm of distortion in the two heads to bring the two tails together in S2 (3). We don’t know how this distortion is distributed between the heads but as shown in the diagram the strain in the structure is likely to operate in opposite directions on the two heads, in the direction of the power stroke for the trailing head and in opposite direction for the lead head. If the movement associated with ADP dissociation is in the same direction as the power stroke (Fig. 5A), then ADP binding to the lead head will reduce the strain in the system whereas binding an ADP to the trailing head will tend to increase the strain. It is expected therefore that the lead head will have a higher affinity for ADP and that the first ADP to bind will go to the lead head. C, the two heads of acto-HMM are represented by the two A.Ms joined by a line, with the trailing head to the left of the two and ADP represented by D. Starting from the position with both heads in rigor (pyrene fluorescence quenched) ADP binds to the lead head in step 1. It can then either bind a second ADP with lower affinity to the trailing head (step 2a) followed by detachment of one head in step 3a (probably the lead head because this is strained toward a state with lower actin affinity). Alternatively the lead head detaches (step 2b) followed by binding of the second ADP (3b). Considering only the route through steps 2a and 3a, then at saturation with ADP, 50% of heads are A.M.D (producing the slow phase of the pyrene signal change on adding ATP) and 50% are M.D giving no pyrene signal change on adding ATP. At saturation the predominant state is therefore the A.M.D-M.D state with little occupancy of A.M.D-A.M.D. Therefore \[ \frac{[A.M.D-M.D]}{[A.M.D-A.M.D]} = K_{s1} \approx 10 \] (because the occupancy of A.M.D-A.M.D is small) and the lower affinity of ADP for the second head is defined by \[ K_{s1} = 10^4 \text{ M}^{-1} \] and \[ K_{s2} = 10^5 \text{ M}^{-1} \]. In the presence of 16 \( \mu \text{M} \) ADP the first head will be close to saturation \( K_{s2} = 10^5 \text{ M}^{-1} \) and we observe equal amplitudes of fast and slow phase (Fig. 4D) at which stage the total amplitude has fallen by 10–20%. Thus the head occupancy must be 20% M.D (no signal), 40% A.M (fast phase), and 40% A.M.D (slow phase). The above set of values for the equilibrium constants fits this data (-10% A.M.A.M, 35% A.M.A.M.D, <5% A.M.D-A.M.D, 40% A.M.D-M.D). In the other case, consider the reaction going possible to tell if phosphorylation affects the binding to actin. However, it is clear that in the absence of nucleotide both heads of thioP-HMM and uP-HMM bind stoichiometrically to actin and form the normal rigor-like R-conformation of the actomyosin complex that results in quenching of the pyrene fluorescence. In the presence of ADP, the affinity of thioP-HMM for actin (18 nM) was ~2 times tighter than that for recombinant smooth S1 under similar conditions (Table I, proteolytically prepared smooth S1 was 87 nM (11)). The contribution of the second head is expected to produce higher affinity binding. In a study of skeletal S1 versus HMM, Conibear and Geeves (3) found that HMM bound to actin in the presence of ADP at 20–50 times the affinity of S1 (depending upon the ionic strength), so the 2-fold difference observed here is small. A key observation of the work on skeletal HMM showed that in the presence of ADP the probability of the myosin forming an R-like conformation was significantly reduced for HMM compared with S1. This was most simply observed by adding ADP to a saturated actin-HMM complex where an increase in pyrene fluorescence was observed without any detachment of HMM from actin indicating loss of ~20% of the R-states. This was suggested to be due to strain induced between the two heads in trying to form identical stereospecific interactions with two actin sites 5.5 nm apart (see Fig. 5). This effect of strain was not apparent in the absence of nucleotide because the binding was very tight. The affinity of smooth HMM for actin is tighter than that for skeletal HMM, it is weakened only 5-fold by ADP compared with >20-fold for skeletal S1, and there is no apparent loss of R-states upon addition of ADP to the thioP-HMM. It appears therefore that both heads can form the R-state interaction, but the second head makes only a modest contribution to the overall affinity.

The situation with uP-HMM in the presence of ADP is markedly different. The affinity of uP-HMM for actin (51 nM) is significantly weaker than that of thioP-HMM (18 nM) and close to that of S1 (49 nM) suggesting very little contribution of the second head to overall affinity. Furthermore despite a 1:1 stoichiometry between actin and HMM only half of the heads are in the R-state. This result is supported by the maximum fluorescence amplitude data and the amplitudes observed on adding excess ADP to acto-uP-HMM (Figs. 1 and 2). It is significant that the fluorescence transient observed on addition of ADP to acto-uP-HMM is very slow and consistent with a slow first order rearrangement event and not ADP binding itself. The stoichiometry implies that the second head of a molecule is bound to actin but not in an R-state or that it is detached but in a nearby position that prevents binding of another head to that site. A structure similar to that observed by Wendt et al. (5) for uP-HMM (in the presence of ATP and the absence of actin), in which only one head of HMM is available to bind to actin, is compatible with the data presented here.}

Affinity of ADP for HMM—The binding of ADP to thioP-
HMM was consistent with two identical binding sites with an affinity of 0.7 μM, which was reduced to 10 μM in the presence of actin. These results are similar to those of smooth S1 (1 μM without actin, 5 μM in the presence of actin) and suggest that under all of the conditions examined here the two nucleotide sites bind ADP independently of each other. The observation that the ADP affinity is reduced 5–10-fold upon actin binding for both S1 and thioP-HMM is consistent with the two independent heads binding to actin in the same way.

For uP-HMM, in the absence of actin there is evidence that ADP binds to two classes of binding sites with affinities of 2 μM and >40 μM. It is possible that the two classes of binding sites are related to the asymmetric structure of uP-HMM observed in two-dimensional crystals such that one of the two sites is blocked by the adjacent head (5).

In the presence of actin there is also evidence of two classes of binding sites for ADP with 10-fold different affinities (1–2 and 10–20 μM). The first affinity is similar to the affinity of ADP for an actin-free S1 head (1 μM), and the second is similar but weaker to the affinity of ADP for acto-S1 or acto-thioP-HMM (5–10 μM). At a simple level this is compatible with only one head being attached to actin, but several issues need to be explained. Why does only one head reduce its affinity for actin dramatically? Given that both heads of HMM are bound tightly to actin in the absence of nucleotide (pyrene fluorescence quenched), how can the affinity of one head of HMM for ADP be so tight? Why is the affinity of ADP for the second head (10–20 μM) weaker than for acto-S1 or acto-thioP-HMM (5 μM)?

**Strain Model—**To interpret the data on actin binding to uP-HMM in more detail we need to consider the interplay of mechanical distortion between the two heads and ADP binding. Consider the two major changes in conformation of acto-S1 that are thought to occur for smooth S1 on release of Pi and ADP as shown in Fig. 5A. If the two heads of HMM bind in the rigor conformation to two adjacent actins that are 5.5 nm apart in the filament, then there must be 5.5 nm of distortion in the two heads to bring the tails together in the subfragment-2 region of the rod. How this distortion is distributed between the heads is not known, but as shown in Fig. 5B, the strain in the structure is likely to operate in opposite directions on the two heads: in the direction of the power stroke for the trailing head and in the opposite direction for the lead head.

If the movement associated with ADP dissociation is in the same direction as the power stroke (Fig. 5A) then ADP binding to the lead head will reduce the strain in the system, whereas binding an ADP to the trailing head will tend to increase the strain. It is expected therefore that the lead head will have a higher affinity for ADP and that the first ADP to bind will go to the lead head. In the case of thioP-HMM these effects must be small because no difference between the ADP binding to the two heads is observed. We can now consider the model shown in Fig. 5C for uP-HMM where the two M’s joined by a line represent the two heads of HMM and the trailing head is to the left of the two.

Starting from the position with both heads in rigor (pyrene fluorescence quenched) ADP binds to the lead head (step 1). It can then either bind a second ADP with lower affinity to the trailing head (step 2a) followed by detachment of one head (probably the lead head because this is strained toward a state with lower actin affinity) (step 3a). Alternatively the lead head detaches (step 2b) followed by binding of the second ADP (step 3b). Both the binding of ADP to the lead head and the detachment of the leading head will reduce the strain on the trailing head and thereby make ADP binding easier to the second head leading to increased affinity for ADP. In step 1, the affinity of ADP is of the order of 1 μM, which is tighter than seen for acto-thioP-HMM or acto-S1, 5 μM and similar to an actin-free head. The higher affinity is a result of the loss of strain within the system on binding ADP. Detailed consideration of the route through the reaction scheme cannot distinguish which route will be preferred but the experimental data suggest (see legend to Fig. 5C) the occupancy of AM-MD or AMD-AMD to be small at intermediate ADP concentrations the predominant states to be AM-AMD and AMD-MD. Thus binding of ADP to the trailing head is required to produce significant detachment of the lead head. It is likely that the conformational change of the rear head on binding ADP, which moves the neck away from the lead head, contributes to the detachment of the lead head. Such coupled movements of the two heads in relation to either P or ADP release could provide a mechanism of gating the response of the two heads or of processivity under the correct conditions as seen for the kinesin family of motors (16–18).

The observation that this behavior is seen only in the uP-HMM and not in the thioP-HMM suggests that phosphorylation changes the mechanical coupling between the two heads. The role of such mechanical effects in regulation of smooth HMM activity or the latch state remains to be determined.

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**REFERENCES**

1. Trybus, K. M., Freyman, Y., Faust, L. Z., and Sweeney, H. L. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 48–52
2. Cremo, C. R., Sellers, J. R., and Facemyer, K. C. (1995) *J. Biol. Chem.* 270, 2171–2175
3. Conibear, P. B., and Geeves, M. A. (1998) *Biophys. J.* 75, 926–937
4. Hackney, D. D., and Clark, P. K. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 5345–5349
5. Wendt, T., Taylor, D., Messier, T., Trybus, K. M., and Taylor, K. A. (1999) *J. Cell Biol.* 147, 1385–1390
6. Deleted in proof
7. O’Reilly, D. R., Miller, L. K., and Luckow, V. A. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*, Freeman and Co., NY
8. Spudich, J. A., and Watt, S. (1971) *J. Biol. Chem.* 246, 4866–4871
9. Criddle, A. H., Geeves, M. A., and Jeffries, T. (1985) *Biochem. J.* 232, 343–349
10. Kurnawwa, S. E., and Geeves, M. A. (1996) *J. Muscle Res. Cell Motil.* 17, 669–674
11. Cremo, C. R., and Geeves, M. A. (1998) *Biochemistry* 37, 1969–1978
12. Rosenfeld, S. S., Xing, J., Cheung, H. C., Brown, F., Kar, S., and Sweeney, H. L. (1998) *J. Biol. Chem.* 273, 28682–28690
13. Bennett, A. J., and Bagshaw, C. R. (1986) *Biochem. J.* 233, 179–186
14. Woodward, S. K., Eccleston, J. F., and Geeves, M. A. (1991) *Biochemistry* 30, 422–430
15. Greene, L. E. (1981) *Biochemistry* 20, 2120–2126
16. Gilbert, S. P., and Johnson, K. A. (1994) *Biochemistry* 33, 1951–1960
17. Cross, K. A. (1989) *Curr. Biol.* 9, R54–R58
18. Ma, Y. Z., and Taylor, E. W. (1997) *J. Biol. Chem.* 272, 724–730
19. Rayment, I., Holden, H. M., Whitaker, M., Yohn, C. B., Lorenz, M., Holmes, K. C., and Milligan, R. A. (1993) *Science* 261, 58–65
20. McKillop, D. F., and Geeves, M. A. (1990) *Biochem. Soc. Trans.* 18, 585–586
21. Whitaker, M., Wilson-Kubalek, E. K., Smith, J. E., Faust, L., Milligan, R. A., and Sweeney, H. L. (1995) *Nature* 378, 748–751