Adenosine diphosphate and strain sensitivity in myosin motors

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The release of adenosine diphosphate (ADP) from the actomyosin cross-bridge plays an important role in the adenosine-triphosphate-driven cross-bridge cycle. In fast contracting muscle fibres, the rate at which ADP is released from the cross-bridge correlates with the maximum shortening velocity of the muscle fibre, and in some models the rate of ADP release defines the maximum shortening velocity. In addition, it has long been thought that the rate of ADP release could be sensitive to the load on the cross-bridge and thereby provide a molecular explanation of the Fenn effect. However, direct evidence of a strain-sensitive ADP-release mechanism has been hard to come by for fast muscle myosins. The recently published evidence for a strain-sensing mechanism involving ADP release for slower muscle myosins, and in particular non-muscle myosins, is more compelling and can provide the mechanism of processivity for motors such as myosin V. It is therefore timely to examine the evidence for this strain-sensing mechanism. The evidence presented here will argue that a strain-sensitive mechanism of ADP release is universal for all myosins but the basic mechanism has evolved in different ways for different types of myosin. Furthermore, this strain-sensing mechanism provides a way of coordinating the action of multiple myosin motor domains in a single myosin molecule, or in complex assemblies of myosins over long distances without invoking a classic direct allosteric or cooperative communication between motors.

Keywords: actin; actomyosin; muscle; cross-bridge cycle; Fenn effect

1. INTRODUCTION

The release of ADP from the actomyosin cross-bridge plays an important role in the ATP-driven cross-bridge cycle. In fast-contracting muscle fibres, the rate at which ADP is released from the cross-bridge correlates with the maximum shortening velocity of the muscle fibre, and in some models the rate of ADP release defines the maximum shortening velocity (Siemankowski *et al.* 1985; Pereira *et al.* 2001; Weiss *et al.* 2001). In addition, it has long been thought that the rate of ADP release could be sensitive to the load on the cross-bridge and thereby provide a molecular explanation of the Fenn effect (Fenn 1923). However, direct evidence of a strain-sensitive ADP-release mechanism has been hard to come by for fast muscle myosins. The recently published evidence for a strain-sensing mechanism involving ADP release for slower muscle myosins, and in particular non-muscle myosins, is more compelling (Coluccio & Geeves 1999; Veigel *et al.* 2003; Khromov *et al.* 2004), and is at the heart of models of myosin V processivity (Rief *et al.* 2000; Mehta 2001; Moore *et al.* 2001; Veigel *et al.* 2002). It is therefore timely to examine the evidence for this strain sensing mechanism. The evidence presented here will argue that a strain-sensitive mechanism of ADP-release is universal for all

myosins but the basic mechanism has evolved in different ways for different types of myosin. Furthermore, this strain-sensing mechanism provides a way of coordinating the action of multiple myosin motor domains, in a single myosin molecule or in complex assemblies of myosins, over long distances without invoking a classical direct allosteric or cooperative communication between motors.

The simplest mechanical version of the cross-bridge cycle is shown in figure 1, which is essentially the Lymn & Taylor (1971) cross-bridge cycle with the addition of a strained cross-bridge (figure 1*b*). The cross-bridge is shown, for simplicity of drawing, with an elastic link between the cross-bridge and the thick filament. When the cross-bridge goes through its powerstroke (on P_i release) while attached to a load, the cross-bridge becomes strained and the strain can be released by the relative sliding of the two filaments (figure 1*b,c*). The speed of sliding will depend upon the load and the force generated by the myosin cross-bridge(s). For the energy produced by the ATP hydrolysis to be used efficiently the cross-bridge should not bind ATP and detach from actin until the sliding of the filaments is complete. Equally, if the muscle is working under isometric conditions, to stabilize posture for example, then a more efficient usage of ATP is achieved if the strained cross-bridge, (figure 1*b*), has a long lifetime. This can simply be achieved if the rate of ADP release from (or ATP binding to) the strained cross-bridge is slowed (figure 1*b* to *d*) relative to the unstrained cross-bridge (figure 1*c* to *d*) in which the relative sliding is complete.

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One contribution of 14 to a Discussion Meeting Issue 'Myosin, muscle and motility'.

Figure 1. A mechano-chemical cross-bridge cycle for myosin. The cross-bridge cycle is shown essentially as described by Lymn & Taylor (1971) but with a strain-dependent cross-bridge detachment step. The ATPase cycle is shown as states (a)–(e) in cartoon and biochemical states (A, actin; M, myosin head; T, ATP; D, ADP). The orange circles represent individual actin sites, the myosin motor domain is shown in green and the light chains in yellow. The adenosine is shown in blue attached to two or three phosphates in red or yellow after hydrolysis. The only significant difference to the Lymn and Taylor model is that the loss of P_i is shown as the result of the development of a strain between the two filaments, which is shown for simplicity as an elastic element connecting the motor domain to the thick filament. ADP release can occur from either state (b) (the strained A·M[#]·D state) or state (c) A·M·D, the state in which the strain is relieved by a relative sliding of the two filaments.

Direct evidence for strain-limited ADP-release (or ATP binding) has been hard to come by for fast muscle myosins (but see Dantzig *et al.* 1991). However, it is clear that ADP release can be important in timing cross-bridge detachment. Siemankowski *et al.* (1985) argued that for a muscle, where multiple cross-bridges are contributing to the observed contraction, the maximum velocity at which a muscle could contract, V_o , is defined by the lifetime of the attached cross-bridge, τ , and the cross-bridge working distance, d , ($V = d/\tau$). They argued that the lifetime of the cross-bridge is controlled by the rate of cross-bridge detachment and this is in turn defined by the rate of ADP release, k_{AD} , ($k_{AD} = 1/\tau$).

Under the conditions of high velocity, the cross-bridge will experience low loads and the ADP release rate will be expected to approach that of free myosin heads in solution (assumed to be zero load). This is indeed what was observed by White and colleagues (Siemankowski *et al.* 1985). For a wide range of muscle myosins (chicken smooth, bovine cardiac and rabbit fast skeletal muscle) they found a very good correlation between the maximum velocity of shortening of the muscle and the rate constant of ADP release. We have found a similar correlation between velocity and the affinity of ADP for actomyosin when examining four adult rat skeletal muscle myosin isoforms (Weiss *et al.* 2001; figure 2).

This shows that the properties of these closely related myosin heads have been 'tuned' such that ADP release is adjusted to match the physiological needs of the muscle. By a detailed analysis of a wider set of muscle myosin isoforms (e.g. paralogous myosin isoforms within a single organism as shown in figure 2 for the rat and the same myosin isoform across a group of closely related mammals e.g. mouse, rat, rabbit, human), it may be possible to correlate changes in ADP release with changes in myosin sequence.

The above studies are under low load conditions. Here we are concerned with how the rates and equilibrium

Figure 2. The correlation between the velocity of shortening and affinity of ADP for actomyosin. The maximum shortening velocity (V_o , muscle length L s^{-1}) of a single muscle fibre was measured for single-skinned rat muscle fibres expressing a single myosin heavy-chain isoform. The same myosin heavy-chain isoforms were isolated and the dissociation constant of ADP for actomyosin (K_{AD}) was measured in solution. Data from Weiss *et al.* (2001), with permission from the authors. The mean values are plotted with the error bars representing the standard deviation.

constants of ADP release are affected by load and the molecular mechanism that underlies such a strain-sensing behaviour.

The simplest idea for how ADP release could be coupled to the load on the cross-bridge is that there is a conformational change in the myosin head which is required before ADP can be released, and this change in conformation is opposed by the load on the cross-bridge. Smith & Geeves (1995) incorporated such a conformational change when modelling the effect of load on ADP release in muscle

Figure 3. A model for the strain-dependent ADP release mechanism. The figure shows the steps following the powerstroke of actomyosin which is coupled to the release of phosphate ($\text{A} \cdot \text{M} \cdot \text{D} \cdot \text{P}_i$ to $\text{A} \cdot \text{M} \cdot \text{D}$). This is shown in cartoon form as a bend of the myosin head. The powerstroke is followed by an isomerization of the cross-bridge which bends the head yet further in the same direction as that caused by the powerstroke. As part of this process the ADP pocket is opened and in the following step ADP is released. The equilibrium constant for the step that opens the pocket is K_{CO} and that for the ADP release is K_{ADP} . The model is based on those developed by Smith & Geeves (1995) for muscle myosin II and for rat myosin I by Coluccio & Geeves (1999) and Geeves *et al.* (2000).

contraction. The essential idea is shown in figure 3 in the form envisaged for rat liver myosin Ib (also known as myr1, and M1¹³⁰ (Geeves *et al.* 2000)). In this model, the large free energy associated with P_i release drives the powerstroke of the cross-bridge (a swing of the converter and neck of the cross-bridge) to generate force. This is followed by a further swing of the converter and neck of the cross-bridge in the same direction as the powerstroke. This additional swing of the neck must be complete before the ADP binding pocket opens to allow ADP to escape. Such a mechanism provides a simple, elegant way of coupling ADP release to the load on the head. How much the ADP release is affected by the load depends upon the stiffness of the cross-bridge, the size of the conformational change and the change in free energy associated with ADP release. We will consider these effects in more detail below.

Evidence for such a change in conformation coupled to ADP release in fast or slow striated muscle myosins is sadly lacking. However, for smooth and non-muscle myosins there is clear evidence for such a conformational change. An ADP-induced change in the position of the converter and neck region of brush border myosin I and smooth muscle myosin subfragment 1 was reported by Milligan and co-workers (Jontes *et al.* 1995; Whittaker *et al.* 1995) and similar results have been reported for other non-muscle myosins since (see figure 4 and Wells *et al.* 1999; Veigel *et al.* 2002). However, there have been no reports of such a conformational change in a fast myosin (Rosenfeld *et al.* 2000). We argue that despite this lack of evidence, the ADP tail swing is universal for all myosins: it is just harder to see for the fast muscle myosins.

The myosins that show this ADP-linked conformational change in the EM are ones that have been proposed to have evolved for tension bearing and tension sensing mechanisms and therefore this property is more highly developed. Myosins in this category show four properties that may be required for a highly developed strain sensing behaviour:

the ADP-induced tail swing in the EM discussed above; a double step in the laser trap; a small free energy change associated with ADP release from actomyosin; and a weak coupling between the affinity of myosin for ADP and actin ($K_{\text{AD}}/K_{\text{D}}$).

2. DOUBLE STEP IN THE OPTICAL TRAP

A group of myosins (similar to those with the ADP induced tail swing) have been shown in the elegant work of Veigel *et al.* (1999, 2002, 2003), to exhibit a double step in the laser trap, single molecule displacement measurements. These two steps have been interpreted to reflect a displacement step associated with P_i release and a second (usually smaller) displacement step associated with ADP release. Furthermore, a strain dependence of the timing of the second displacement, thought to be ADP release, has been observed. The double step has not been seen in the laser trap for fast myosins. It should be noted that the direct association of the optical trap events with sequential product release have not been fully established. Such measurements are discussed in detail elsewhere in this volume (Batters *et al.* 2004b).

3. SMALL FREE ENERGY ASSOCIATED WITH ADENOSINE DIPHOSPHATE RELEASE

All of the actomyosins that have been shown to have the ADP-induced tail swing have a relatively tight affinity for ADP ($K_{\text{d}} < 20 \mu\text{M}$; table 1). This is comparable to cellular ADP concentrations of 10–50 μM and means that in the cell ADP release occurs with a small (or even positive) free energy change and thus ADP release is unable to contribute directly to work production. By contrast, fast muscle actomyosins have ADP affinities of more than 100 μM , a value higher than cellular ADP concentration and thus ADP release occurs with a significant and negative change in ΔG .

Table 1. The classification of myosin according to ADP affinity and thermodynamic coupling between ADP and actin affinity. (Dd, *Dictyostelium discoideum*; mm, mammalian; ND, not determined; UP1, M. A. Geeves and D. J. Manstein, unpublished data; UP2, C. E. Berger, K. M. Trybus and M. A. Geeves, unpublished data.)

	myosin	K_{AD} (μ M)	K_{AD}/K_D	k_{-AD}/k_{-D}	reference
fast mover	fast skeletal muscle	200	<i>ca</i> 100	>100	Batra <i>et al.</i> (1999)
	slow skeletal and cardiac	100	<i>ca</i> 50	100	Siemankowski & White (1984)
	Dd myosin II	200	<i>ca</i> 50	>20	Batra <i>et al.</i> (1999)
	Dd myosin IB	100	<i>ca</i> 50		UP1
strain sensor	myosin IIa		0.7	3	Kovacs <i>et al.</i> (2003)
	myosin IIb		0.2	0.7	Wang <i>et al.</i> (2003)
	smooth muscle gizzard	5	5	12	Cremo & Geeves (1998)
	smooth muscle aorta	20	<i>ca</i> 1		UP2
	mm myosin Ib	5	<i>ca</i> 5	ND	Geeves <i>et al.</i> (2000)
	mm myosin Ic	5	<i>ca</i> 5	ND	Batters <i>et al.</i> (2004a)
	Dd myosin IE	10	<i>ca</i> 5		UP1
	myosin V	5	<i>ca</i> 3	12	De La Cruz <i>et al.</i> (1999)
	myosin VI	5	<i>ca</i> 1.5	1.1	De La Cruz <i>et al.</i> (2001)

4. WEAK COUPLING BETWEEN ACTIN AND ADENOSINE DIPHOSPHATE BINDING

The idea that nucleotide and actin allosterically displace each other from myosin is well established and expressed in the Lymn–Taylor mechanism (and further developed by Geeves *et al.* (1984)). However, although this appears universally true for ATP (ATP and actin both weaken each other's affinity more than 1000-fold), it is not the case for ADP. For fast muscle myosins, ADP and actin both reduce each other's affinity by *ca* 100-fold while for the group of myosins that show an ADP-linked conformational change, the effect is 5 or less and can be 1 or less (see table 1). The affinities of ADP and actin for myosin are linked by the thermodynamic box shown in figure 5. Detailed balance around this box means that if actin weakens the affinity of myosin for ADP, then ADP must weaken the affinity of myosin for actin to the same degree. This can be expressed by a thermodynamic coupling constant which relates the affinity of ADP for myosin (K_D) and actomyosin (K_{AD}) with the affinity of actin for myosin (K_A) and myosin:ADP (K_{DA}):

$$K_{AD}/K_D = K_{DA}/K_A. \quad (4.1)$$

This ratio is referred to as the thermodynamic coupling constant as it is related to the ratio of the standard free energy changes for ADP binding to actomyosin and to myosin ($\Delta G^\circ_{\{ADP \text{ binding to A.M}\}}/\Delta G^\circ_{\{ADP \text{ binding to M}\}} = \ln(K_{AD})/\ln(K_D)$) and similarly for the free energy change of actin binding to M and to M·D).

This coupling constant is always small (less than 5) for the group of myosins showing the ADP-induced tail swing, whereas for fast muscle myosin this is large (more than 50; table 1). The small value of this coupling is significant not just because the ADP remains tightly bound to myosin in the presence of actin. It also means that the M·D complex is bound to actin almost as tightly as the A·M complex (five times weaker at most) a property that is important for a load-bearing cross-bridge: the myosins are less likely to be forcibly detached by large loads.

There are thus two groups of myosins: the slower myosins that show evidence of an ADP coupled isomerization which can produce movement of the converter and neck; and the fast myosins for which there is currently no firm evidence for such a conformational change. There are far

more data available on the biochemical properties of different classes of myosin than there are from EM or optical trap data. Table 1 lists the myosins for which there is biochemical evidence on the ADP affinity and the coupling of ADP and actin affinities. The table also shows that the kinetic coupling—the degree to which actin accelerates ADP release—is similar but not the same as the thermodynamic coupling of affinities. As shown, all of the myosins fall into one of two categories: what we will call the tension sensors, which have been shown to have the biochemical properties of low ADP and actin thermodynamic coupling; and the fast movers, including skeletal and cardiac muscle myosins. None of the fast movers has so far been shown to have a detectable tail movement on binding ADP in the EM or a double step in the laser trap. By contrast, all of the myosins in the second group that have been tested have these properties.

The importance of tight ADP affinity for actomyosin can be understood in terms of the free energy associated with ADP release and the mechanical state of the cross-bridge for the model shown in figure 3. ADP release in solution is associated with a free energy change, ΔG° , where

$$\Delta G^\circ = -RT \ln K'_{AD}. \quad (4.2)$$

It is more convenient in the working cross-bridge to define the equilibrium constant as the ratio of the equilibrium constant and the cellular ADP concentration as this reflects the free energy available from ADP release.

$$K'_{AD} = K_{AD}/[ADP].$$

If ADP release from the cross-bridge is associated with a movement against an immovable load then ADP release requires work to be done to stretch the elastic spring. For a classical spring the energy of stretching is given by

$$\text{energy} = +k\Delta L^2/2, \quad (4.3)$$

where k is the spring constant and ΔL is the size of the imposed stretch.

The net free energy of ADP release is modified by the energy required to stretch the spring (see figure 6):

$$\text{net energy change, } \Delta G = \Delta G^\circ + k\Delta L^2/2. \quad (4.4)$$

Note, in the direction of the reaction as drawn in figure 6

the sign of the free energy changes are opposite for ADP release and the spring stretching.

The apparent equilibrium constant (K'_{AD}) of ADP release under these conditions is then defined by

$$\Delta G = -RT \ln K'_{AD} + k\Delta L^2/2 = -RT \ln K''_{AD}, \quad (4.5)$$

$$\text{and } K''_{AD} = K'_{AD} \exp(-k\Delta L^2/2RT). \quad (4.6)$$

Numerical examples help to illustrate the significance of these equations. If $k = 10^{-3} \text{ N m}^{-1}$ then we can calculate the size of the displacement required to reduce ΔG in equation (4.5) to zero, i.e. when K''_{AD} has a value of 1. This occurs for ΔL of 1, 2, 3 and 5 nm when K'_{AD} is *ca* 2, 10, 100 and 10^5 . This illustrates the nonlinearity of the relationship between the parabolic strain and the logarithmic K'_{AD} .

We can consider three cases for the relationship between the free energy of ADP release and movement against a load. If the free energy of ADP release is negative and large compared with the elastic energy then ADP release could contribute towards force generation and movement. If the free energy change of ADP release is small (*ca* 0) then modest loads on the head will have a marked effect on the apparent ADP affinity and therefore most probably on the net rate of ADP release. The ADP release from myosin can then act as a strain sensor. If the free energy change of ADP release is large and positive the ADP is trapped on the head. Not only will load increase the trapping of ADP, the efficient release of ADP will require the input of free energy. If the energy needed is large, it is unlikely to come from thermal energy, and ADP release can act as a gate. ADP release is only possible with the intervention of another factor. This is analogous to the situation for G-proteins where the guanosine diphosphate remains trapped on the protein until another signal is received: for example the binding of the guanine nucleotide exchange protein, GEF. In the case of the mechanical model for myosin, the signal for ADP release could come from a reduction in the load, a change in the elasticity of the spring or the contribution of a second myosin sharing the load. Each of these could reduce the free energy of ADP release sufficiently to allow productive ADP release.

The comparison between elastic free energy and ADP binding or releasing free energy can be usefully considered by using the parabolic free energy diagrams introduced by Hill (1974). The minimum energies of the parabolas are defined by the free energy differences between the unconstrained intermediates (ΔG^0 and equation (4.4)). The position of the minima in the x direction is defined by the position of minimum energy of the spring (by convention the spring is assumed to be symmetrical and can be stretched or compressed). The free energy of any attached actomyosin state then depends upon the distance away from the minima of the spring (ΔL) and is described by a parabola as in equation (4.5). In such a diagram, the powerstroke and the accompanying release of P_i is shown as a pair of parabolas (A·M·D· P_i and A·M·D), displaced in the x direction by the distance of the cross-bridge throw (here assumed to be 5 nm), and in the vertical direction by the net free energy of P_i release (see figure 7).

In the case of a fast-contracting muscle where there is no detectable change in structure on ADP release, the A·M and A·M·D parabolas are just displaced vertically and the change in free energy on ADP release is independent of x or

the load already borne by the head. This is true whether ADP release occurs with zero (A·M curve superimposed on A·M·D), positive (A·M⁺) or negative (A·M⁻) change in free energy (dotted lines in figure 7a).

If the powerstroke accompanying P_i release occurs with a 5 nm displacement and ADP release produces an additional displacement of 2 nm in the same direction, we have the situation in figure 7b (as seen in smooth muscle S1 (Cremo & Geeves 1998)). Here the important point is the place at which the A·M·D and A·M parabolas cross and this is a function of the free energy of ADP release and the stiffness of the elastic element (assumed to be 1 pN nm^{-1}). If, for the unstrained system, ADP release occurs with zero free energy change then the parabolas cross at a displacement of *ca* 6 nm. Thus, even if the P_i release driven powerstroke and movement go to completion, (5 nm displacement to the minima of the A·M·D curve) there is still a need to put work into the system to allow ADP to be released (this may be possible from thermal energy depending on the nature of the transition state, see below). Note that the difference between the A·M and A·M·D curves is a linear function of x .

The dotted lines show the situation if ADP release in the unstrained system occurs with a small ($= 1 RT$), increase or decrease in free energy. These have the effect of moving the crossover point of the parabolas *ca* 1–1.5 nm to the left or right. ADP release would need to occur with a very large negative free energy change to allow ADP release from a bridge holding a significant amount of the isometric load.

If the ADP displacement requires an even larger step, 6 nm for the P_i coupled powerstroke and a further 5 nm for ADP release, then the situation is shown in figure 7c (the situation modelled for myosin Ib (Coluccio & Geeves 1999; Veigel *et al.* 1999)). Here the crossover point is at *ca* 7 nm and the energy required to go from A·M·D to A·M is large for any displacement of less than 5 nm. Thus the ADP release is unlikely to happen until the powerstroke and displacement is almost complete. Even modest strains will tend to trap the ADP in the pocket. Again note the diagram is drawn for zero free energy change on ADP release from the unconstrained head.

If the ADP release occurs with a small free energy change then this ADP linked tail movement provides a mechanism to trap ADP on any myosin bearing a load. It is therefore important that a myosin with a strain-sensing behaviour has an ADP affinity of the same order as the ADP concentration in the cell. Fluctuations of global or local cellular ADP concentration could play a part in modulating the sensitivity of the system to load.

A more extreme case of trapped ADP may be that found in latch and catch states of actomyosin cross-bridges which can hold tension for very long time periods with zero energy turnover. The escape from such a state can be facilitated by the action of an additional agent, such as calcium binding to the calmodulin or light chains of the myosin neck, to reduce the stiffness of the cross-bridge and therefore lowering the energy required to release ADP. Alternatively another protein could bind to reduce the affinity of actomyosin for ADP as the exchange factors do for G-proteins.

Figure 4. The ADP-induced movement of the light-chain binding domain of myosin 1. Three-dimensional maps of mammalian myosin IC bound to actin were calculated by cryo-EM and image analysis. Actin is on the right running vertically, with the pointed end of the filament at the bottom. The crystal structure of chicken skeletal myosin (wire format) is superimposed onto the density with three light chains. The images show the maps of the structure in the presence of ADP (top, blue) and absence of ADP (middle, red) and the two images superimposed (bottom panel). The removal of ADP causes little change in the motor domain but the light chain tail tilts by 33° in the same direction as the powerstroke. Image courtesy of Dr R. A. Milligan based on Batters *et al.* (2004a).

Figure 5. The thermodynamic coupling between ADP and actin binding to myosin. M, A and D symbolize the myosin, actin and ADP, respectively. The dissociation constant of the myosin for actin is characterized by K_A in the absence of nucleotide and by K_{DA} in the presence of ADP. The dissociation constant of myosin for ADP is given by K_D and K_{AD} in the absence and presence of actin, respectively.

5. MYOSIN IB AND TWO FORMS OF A·M AND A·M·D

The clearest evidence for a nucleotide pocket opening by actomyosin comes from a study of ATP and ADP binding

to mammalian actomyosin Ib. The essential observation of Geeves *et al.* (2000) is shown in figure 8. The ATP-induced dissociation of myosin from pyrene-labelled actomyosin was seen to comprise two phases. A fast phase which, like all other myosins studied, shows a hyperbolic dependence on ATP concentration indicative of a two step reaction. This comprises a rapid reversible binding of ATP to A·M followed by a rate-limiting change in conformation which results in a change in pyrene fluorescence signal and dissociation of the actomyosin complex. The second phase was independent of ATP and is most simply interpreted as a fraction of the actomyosin being unable to bind ATP quickly and undergoing a slow isomerization before ATP binds. The relative amplitudes of the two phases define the fraction of A·M existing in the two forms. Addition of ADP titrates away the fast phase leaving all of the A·M in a slow reacting form. Detailed analysis of the data results in the model shown in figure 9.

This suggests an equilibrium between accessible and inaccessible nucleotide binding forms which is equal to 3 in the absence of nucleotide and 0.1 or less in the presence of ADP. Thus access into and out of the nucleotide pocket requires a conformational change of the protein. If this conformational change is related to the tail movement seen

Figure 6. The free energy of ADP release coupled to a mechanical event. The effect of mechanical strain on the net free energy of ADP release shown in (a) for the case where ΔG° for ADP release is greater than the cost of loading a spring and (b) where ΔG° is smaller than the spring energy. The symbols A and M are the same as in figure 5.

on binding ADP in the EM then we have the model of figure 3.

Combining the two-step dissociation of ADP in figure 3 with the free energy diagrams for ADP release (figures 6 and 7) suggests the primary effect of strain is on the isomerization step, not the ADP release itself. The apparent equilibrium constant (K'_{OC}) of the pocket closure under strain, based on the argument of equation (4.5) is then defined by:

$$\Delta G = -RT \ln K_{OC} + k \Delta L^2 / 2 = -RT \ln K'_{OC}, \quad (5.1)$$

$$\text{and } K'_{OC} = K_{OC} \exp(-k \Delta L^2 / 2RT). \quad (5.2)$$

Numerical examples help to illustrate the significance of these equations. If $k = 10^{-3} \text{ N m}^{-1}$ then at 20 °C we can calculate the size of the displacement to reduce ΔG in equation (5.1) to zero, i.e. when K'_{OC} has a value of 1. This occurs for ΔL of 1, 2, 3 and 5 nm when K_{OC} is *ca* 2, 10, 100 and 10^5 . This illustrates the nonlinearity of the relationship between the parabolic strain and the logarithmic K_{OC} .

When the conformation change (open or closure of the pocket) is coupled to the ADP binding or release step (figure 3), we can define the effect of strain on the apparent ADP affinity. The overall equilibrium constant, $K^{\#}_{ADP}$, of ADP binding to the actomyosin is

$$K^{\#}_{ATP} = K_{ADP} K'_{OC} / (1 + K'_{OC}). \quad (5.3)$$

ADP binding or release to the open state (K_{ADP}) is independent of strain but the overall affinity ($K^{\#}_{ADP}$) is coupled to strain through the strain dependence of the open to closed transition and K'_{OC} . Note that the effect of strain decreases as K'_{OC} increases and the affinity of ADP is inde-

pendent of strain for $K'_{OC} (\geq 10)$. For small values of K'_{OC} , the affinity of ADP is linearly dependent upon K'_{OC} and the strain:

$$K^{\#}_{ADP} = K_{ADP} K'_{OC} \cdot \exp(-k \Delta L^2 / 2RT) \quad (5.4)$$

6. RATE CONSTANTS VERSUS EQUILIBRIUM CONSTANTS

The argument for a strain-sensitive ADP release mechanism has been developed by considering only the equilibrium constants for ADP binding and release. In many cases the rate constant for ADP release is of equal significance (the rate constants of ADP binding and release are of course related to the equilibrium constant). However, modelling the effect of strain on the rate constants requires knowledge, or at least a clear set of assumptions, about the free energy landscape between the A·M·D and A·M states. This is beyond the current discussion but the reader is referred to the recent work by Smith & Sleep (2004) who have developed such an approach for the P_i release steps of the cross-bridge cycle. A similar approach is possible for the ADP release steps and will be developed elsewhere.

7. IS THIS A SPECIAL FUNCTION OF CERTAIN TYPES OF MYOSIN?

Is the coupling of pocket opening or closing to nucleotide binding unique to myosin Ib? Recent data have shown the same behaviour in other myosins. Rat myosin Ic (Batters *et al.* 2004a) and a *Dictyostelium* myosin belonging to the same myosin subfamily (myo 1E; D. J. Manstein unpublished data) show essentially the same behaviour; suggesting this is not a property of an unusual myosin but common to myosins of the same subgroup. We propose that it may

Figure 7. The free energy diagrams for an actomyosin cross-bridge subject to a load. The diagrams show the free energy of the biochemical attached states A-M·D·P_i, A-M·D and A-M as parabolas. The free energy change between states measured in solution is represented by the minima of the parabolas. The shapes of the parabolas are defined by the stiffness of the cross-bridge (assumed to be 1 pN nm⁻¹) and the size of the cross-bridge shape changes in each step. (a) The free energy changes for a fast muscle myosin where the crossbridge throw on P_i release is 5 nm and there is no additional change on ADP release. The dotted lines represent the curves for ADP release occurring with either plus (A-M⁺) or minus (A-M⁻) 1 RT of energy. (b) The same diagram drawn for smooth muscle myosin in which ADP release involves an additional 2 nm of movement. ADP release is shown with zero (solid line A-M) or plus or minus 1 RT energy change (A-M⁺, A-M⁻, respectively). (c) The same as (b) but with a larger structural change on ADP release as may be possible for non-muscle myosins with extended light-chain-binding domains.

be common to other myosins designed for strain sensing and tension holding. Such a property may not have

Figure 8. ATP-induced dissociation of pyrene-actin.myosin I. (a) The observed fluorescence transient on displacing myosin from actomyosin by the addition of ATP at the post-mixing concentrations shown. The best fit to the sum of two exponentials is shown superimposed. (b) The dependence of the observed rate constant (k_{obs}) of the two phases seen in (a) on ATP concentration. The k_{obs} of the slow phase (open circles) is independent of ATP concentration whereas the k_{obs} of the fast phase (filled squares) shows a hyperbolic dependence with a maximum rate constant of 79 s⁻¹ and 1.5 mM ATP concentration required for a half maximal observed rate constant. Redrawn from Geeves *et al.* (2000) using the motor and one light chain binding domain of mammalian myosin Ib co-expressed with calmodulin.

developed or may have been suppressed in fast-moving myosins. However, it seems clear that the Fenn effect and the efficiency of muscle contraction is most readily explained by a load-sensitive ADP release step. If the ADP release does occur in two steps, a conformational change followed by ADP release (figure 3), then the difference between fast-moving myosins and the other group could simply be in the value of the equilibrium constant for the isomerization step. This may have a value close to 1 for the strain sensors and a value of greater than 1 for the fast movers. Thus, simple addition of ADP to a fast actomyosin cannot reverse the isomerization step and the tail movement would not be seen in the EM. This is the argument for an additional A-M·D state only accessible during ATP turnover, first proposed by Sleep & Hutton (1980).

This argument does not apply to the optical trap data that were collected under ATP hydrolysing conditions and should result in the two step events. However, so far, the fast-moving myosins all have ADP release rates in excess of

K_{α}	$k_{+\alpha}$	$k_{-\alpha}$	$k_{\alpha D}$	$k_{+\alpha D}$	$k_{-\alpha D}$	k_{ADP}	k_{+ADP}	k_{-ADP}
	s^{-1}	s^{-1}		s^{-1}	s^{-1}	μM	$M^{-1} s^{-1}$	s^{-1}
2–3	8	3–4	≤ 0.1	8	≥ 80	100	fast	fast

Figure 9. A model for the interaction of ADP and ATP with rat myosin Ib. The data from figure 8 together with other measurements can be interpreted in terms of the above model with rate and equilibrium constants as shown. The open and closed nucleotide pockets of myosin can be considered to correspond to the states shown in figure 3. Calcium binds to the calmodulin of the myosin and modifies the rate and equilibrium constants as shown. Redrawn from Geeves *et al.* (2000).

$100 s^{-1}$ and therefore are beyond the resolution of the optical trap data. Faster traps or low temperature studies will be required to test if such events do exist.

8. ADENOSINE DIPHOSPHATE TRAPPING

The above discussion has focused on the presence of a second form of bound ADP in which the ADP is tightly bound to actomyosin. There is evidence that ADP can be trapped on fast muscle myosin by a variety of treatments. Of most significance physiologically is the modification of the light chains via regulatory agents. Removal of calcium from the essential light chain of scallop HMM results in a 15-fold inhibition of ADP release from HMM and a greater than 100-fold inhibition of ADP release from actoHMM (Nyitrai *et al.* 2002). Removal of calcium from the calmodulin light chain(s) of myosin Ib results in an approximate threefold decrease in ADP release from actomyosin Ib (Coluccio & Geeves 1999). ADP release from smooth muscle HMM with phosphorylated light chains is at least threefold faster than from unphosphorylated HMM (Berger *et al.* 2001). There are differing views on the details of how this regulatory mechanism operates but it does reveal a mechanism of linking the conformation of the neck and lever arm with the nucleotide binding pocket. This is distinct from the tail swing coupled to switch II movement and the presence of γ -phosphate in the nucleotide pocket (Geeves & Holmes 1999).

ADP can also be trapped by cross-linking of the essential thiols of vertebrate muscle myosins. The evidence here points to a major conformational change involving switch II, the relay loop and helix and the reactive thiol region (in turn linked to the converter domain and the lever arm movement) being involved in the conformation of the nucleotide pocket. The thiols can be cross-linked effectively in the nucleotide-bound conformation and once cross-linked, the conformational change required for ADP release is inhibited.

This trapping of the nucleotide may be closely related to the trapping of nucleotide seen in *Dictyostelium* myosin II. This myosin lacks one thiol and the cross-linking is therefore not possible (but see Liang & Spudich 1998). Batra *et*

al. (1999) replaced glycine 680 in *Dictyostelium* MII with alanine. The equivalent glycine in the chicken myosin lies between the two thiols which can be cross-linked, and the glycine undergoes a major change in conformation between the M·ATP and M·ADP·P_i forms of myosin (Geeves & Holmes 1999). The addition of the extra methyl group into the hinge is sufficient to disrupt the conformational changes in this region (valine causes an even bigger disruption (Ruppel & Spudich 1996; Friedman *et al.* 1998)). The myosin continues to function as an ATPase but the rates of nucleotide binding and release are reduced about 100-fold. The equilibrium constant of ADP binding to myosin is relatively little affected as is the rate of ATP hydrolysis step. Thus once ATP is bound, hydrolysis proceeds normally but getting nucleotide into and out of the pocket is difficult. Furthermore, actin binds with a similar affinity to the apo myosin and to myosin ADP, suggesting that the 50 kDa cleft is closed in both cases, but the coupling between ADP and actin binding is abolished. ADP binds with the same high affinity to both myosin and to actomyosin.

A similar phenomenon was noted in a truncated *Dictyostelium* myosin (Woodward *et al.* 1995; Kurzawa *et al.* 1997). Truncation of the myosin at residue 761 (which removes the light chain binding domain or lever arm) produces a myosin that, apart from having very low motility, is very similar to the parent myosin. Truncating seven amino acids earlier at residue 754 results in quite different properties, and in particular, the ADP has a high affinity for myosin and is not displaced by actin. This again, like the modifications of the light chains, suggests a direct communication pathway between the nucleotide pocket and the area between the lever arm and the motor domain.

The argument presented here shows that there is evidence of an ADP-coupled conformational change in actomyosin which is pronounced for certain classes of myosin which we have called the strain sensors. Fast-moving myosins do not appear to have the same property but this may simply be that the equilibrium constant for the event is much larger and the rate much faster. The challenge will be to see if it is possible to convert between the two forms of myosin by small changes in the amino acid sequence in key areas of the myosin motor domain.

The ADP release mechanism is at the core of myosin's function as a molecular motor. The rate of ADP release can define the maximum velocity of movement by the motor. The strain-sensing mechanism discussed here allows the efficient coupling of the ATPase cycle with both movement and load-bearing roles of the motor. It also allows the coordination of groups of myosin motors, or the two heads of a myosin, through their coupling to a single load. A myosin that is stalled with a trapped ADP can be released from the trapped state by a second myosin binding to actin and sharing the load. In a processive motor a pull of the lead head may be required to allow release of ADP from the rear head, thereby providing the underlying mechanism of processivity.

We thank S. S. Lehrer, L. M. Coluccio and D. A. Smith for commenting on an earlier version of this manuscript. This work was supported by a European Union grant HPRN-CT-2000-00091 and a Wellcome Trust Programme grant 055841 (to M.A.G.), and by a Hungarian Scientific Research Fund (OTKA) Grant T043103 (to M.N.). M.N. is an EMBO/HHMI scientist.

REFERENCES

- Batra, R., Geeves, M. A. & Manstein, D. J. 1999 Kinetic analysis of *Dictyostelium discoideum* myosin motor domains with glycine-to-alanine mutations in the reactive thiol region. *Biochemistry* **38**, 6126–6134.
- Batters, C., Arthur, C. P., Lin, A., Porter, J., Geeves, M. A., Milligan, R. A., Molloy, J. E. & Coluccio, L. M. 2004a Myo1c is designed for the adaptation response in the inner ear. *EMBO J.* **23**, 1433–1440.
- Batters, C., Wallace, M. I., Coluccio, L. M. & Molloy, J. E. 2004b A model of stereocilia adaptation based on single molecule mechanical studies of myosin I. *Phil. Trans. R. Soc. B* **359**, 1895–1905. (doi:10.1098/rstb.2004.1559)
- Berger, C. E., Fagnant, P. M., Heizmann, S., Trybus, K. M. & Geeves, M. A. 2001 ADP binding induces an asymmetry between the heads of unphosphorylated myosin. *J. Biol. Chem.* **276**, 23 240–23 245.
- Coluccio, L. M. & Geeves, M. A. 1999 Transient kinetic analysis of the 130 kDa myosin I (MYR-1 gene product) from rat liver. A myosin I designed for maintenance of tension? *J. Biol. Chem.* **274**, 21 575–21 580.
- Cremona, C. R. & Geeves, M. A. 1998 Interaction of actin and ADP with the head domain of smooth muscle myosin: implications for strain-dependent ADP release in smooth muscle. *Biochemistry* **37**, 1969–1978.
- Dantzig, J. A., Hibberd, M. G., Trentham, D. R. & Goldman, Y. E. 1991 Cross-bridge kinetics in the presence of MgADP investigated by photolysis of caged ATP in rabbit psoas muscle fibres. *J. Physiol.* **432**, 639–680.
- De La Cruz, E. M., Wells, A. L., Rosenfeld, S. S., Ostap, E. M. & Sweeney, H. L. 1999 The kinetic mechanism of myosin V. *Proc. Natl Acad. Sci. USA* **96**, 13 726–13 731.
- De La Cruz, E. M., Ostap, E. M. & Sweeney, H. L. 2001 Kinetic mechanism and regulation of myosin VI. *J. Biol. Chem.* **276**, 32 373–32 381.
- Fenn, W. O. 1923 A quantitative comparison between the energy liberated and the work performed by the isolated sartorius muscle of the frog. *J. Physiol.* **58**, 175–203.
- Friedman, A. L., Geeves, M. A., Manstein, D. J. & Spudich, J. A. 1998 Kinetic characterization of myosin head fragments with long-lived myosin.ATP states. *Biochemistry* **37**, 9679–9687.
- Geeves, M. A. & Holmes, K. C. 1999 Structural mechanism of muscle contraction. *A. Rev. Biochem.* **68**, 687–728.
- Geeves, M. A., Goody, R. S. & Gutfreund, H. 1984 Kinetics of acto-S1 interaction as a guide to a model for the cross-bridge cycle. *J. Muscle Res. Cell Motil.* **5**, 351–361.
- Geeves, M. A., Perreault-Micale, C. & Coluccio, L. M. 2000 Kinetic analyses of a truncated mammalian myosin I suggest a novel isomerization event preceding nucleotide binding. *J. Biol. Chem.* **275**, 21 624–21 630.
- Hill, T. L. 1974 Theoretical formalism for the sliding filament model of contraction of striated muscle, part 1. *Prog. Biophys. Mol. Biol.* **28**, 267–340.
- Jontes, J. D., Wilson-Kubalek, E. M. & Milligan, R. A. 1995 A 32 degree tail swing in brush border myosin I on ADP release. *Nature* **378**, 751–753.
- Khromov, A. S., Webb, M. R., Ferenczi, M. A., Trentham, D. R., Somlyo, A. P. & Somlyo, A. V. 2004 Myosin regulatory light chain phosphorylation and strain modulate adenosine diphosphate release from smooth muscle myosin. *Biophys. J.* **86**, 2318–2328.
- Kovacs, M., Wang, F., Hu, A., Zhang, Y. & Sellers, J. R. 2003 Functional divergence of human cytoplasmic myosin II: kinetic characterization of the non-muscle IIA isoform. *J. Biol. Chem.* **278**, 38 132–38 140.
- Kurzawa, S. E., Manstein, D. J. & Geeves, M. A. 1997 *Dictyostelium discoideum* myosin II: characterization of functional myosin motor fragments. *Biochemistry* **36**, 317–323.
- Liang, W. & Spudich, J. A. 1998 Nucleotide-dependent conformational change near the fulcrum region in *Dictyostelium* myosin II. *Proc. Natl Acad. Sci. USA* **95**, 12 844–12 847.
- Lymn, R. W. & Taylor, E. W. 1971 Mechanism of adenosine triphosphate hydrolysis by actomyosin. *Biochemistry* **10**, 4617–4624.
- Mehta, A. 2001 Myosin learns to walk. *J. Cell Sci.* **114**, 1981–1998.
- Moore, J. R., Kremmentsova, E. B., Trybus, K. M. & Warshaw, D. M. 2001 Myosin V exhibits a high duty cycle and large unitary displacement. *J. Cell Biol.* **155**, 625–635.
- Nyitrai, M., Szent-Györgyi, A. G. & Geeves, M. A. 2002 A kinetic model of the co-operative binding of calcium and ADP to scallop (*Argopecten irradians*) heavy meromyosin. *Biochem. J.* **365**, 19–30.
- Pereira, J. S., Pavlov, D., Nili, M., Greaser, M., Homsher, E. & Moss, R. L. 2001 Kinetic differences in cardiac myosins with identical loop 1 sequences. *J. Biol. Chem.* **276**, 4409–4415.
- Rief, M., Rock, R. S., Mehta, A. D., Mooseker, M. S., Cheney, R. E. & Spudich, J. A. 2000 Myosin-V stepping kinetics: a molecular model for processivity. *Proc. Natl Acad. Sci. USA* **97**, 9482–9486.
- Rosenfeld, S. S., Xing, J., Whitaker, M., Cheung, H. C., Brown, F., Wells, A., Milligan, R. A. & Sweeney, H. L. 2000 Kinetic and spectroscopic evidence for three actomyosin:ADP states in smooth muscle. *J. Biol. Chem.* **275**, 25 418–25 426.
- Ruppel, K. M. & Spudich, J. A. 1996 Structure-function studies of the myosin motor domain: importance of the 50 kDa cleft. *Mol. Biol. Cell* **7**, 1123–1136.
- Siemankowski, R. F. & White, H. D. 1984 Kinetics of the interaction between actin, ADP and cardiac myosin-S1. *J. Biol. Chem.* **259**, 5045–5053.
- Siemankowski, R. F., Wiseman, M. O. & White, H. D. 1985 ADP dissociation from actomyosin subfragment 1 is sufficiently slow to limit the unloaded shortening velocity in vertebrate muscle. *Proc. Natl Acad. Sci. USA* **82**, 658–662.
- Sleep, J. A. & Hutton, R. L. 1980 Exchange between inorganic phosphate and adenosine 5'-triphosphate in the medium by actomyosin subfragment 1. *Biochemistry* **19**, 1276–1283.
- Smith, D. A. & Geeves, M. A. 1995 Strain-dependent cross-bridge cycle for muscle. *Biophys. J.* **69**, 524–537.

- Smith, D. A. & Sleep, J. 2004 Kinetic analysis of force-generation and phosphate release in muscle: the myosin working stroke is followed by slow release of phosphate. *Biophys. J.* **87**, 442–456.
- Veigel, C., Coluccio, L. M., Jontes, J. D., Sparrow, J. C., Milligan, R. A. & Molloy, J. E. 1999 The motor protein myosin-I produces its working stroke in two steps. *Nature* **398**, 530–533.
- Veigel, C., Wang, F., Bartoo, M. L., Sellers, J. R. & Molloy, J. E. 2002 The gated gait of the processive molecular motor, myosin V. *Nature Cell Biol.* **4**, 59–65.
- Veigel, C., Molloy, J. E., Schmitz, S. & Kendrick-Jones, J. 2003 Load-dependent kinetics of force production by smooth muscle myosin measured with optical tweezers. *Nature Cell Biol.* **5**, 980–986.
- Wang, F., Kovacs, M., Hu, A., Limouze, J., Harvey, E. V. & Sellers, J. R. 2003 Kinetic mechanism of non-muscle myosin IIB: functional adaptations for tension generation and maintenance. *J. Biol. Chem.* **278**, 27 439–27 448.
- Weiss, S., Rossi, R., Pellegrino, M. A., Bottinelli, R. & Geeves, M. A. 2001 Differing ADP release rates from myosin heavy chain isoforms define the shortening velocity of skeletal muscle fibers. *J. Biol. Chem.* **276**, 45 902–45 908.
- Wells, A. L., Lin, A. W., Chen, L. Q., Safer, D., Cain, S. M., Hasson, T., Carragher, B. O., Milligan, R. A. & Sweeney, H. L. 1999 Myosin VI is an actin-based motor that moves backwards. *Nature* **401**, 505–508.
- Whittaker, M., Wilson-Kubalek, E. M., Smith, J. E., Faust, L., Milligan, R. A. & Sweeney, H. L. 1995 A 35 Å movement of smooth muscle myosin on ADP release. *Nature* **378**, 748–751.
- Woodward, S. K., Geeves, M. A. & Manstein, D. J. 1995 Kinetic characterization of the catalytic domain of *Dictyostelium discoideum* myosin. *Biochemistry* **34**, 16 056–16 064.

GLOSSARY

- A·M: actin·myosin complex
 ADP: adenosine diphosphate
 ATP: adenosine triphosphate
 EM: electron microscopy
 HMM: heavy meromyosin
 M·D: myosin·ADP
 M: myosin
 P_i: phosphate