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Engineering *Dictyostelium discoideum* myosin II for the introduction of site-specific fluorescence probes

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Abstract

Dictyostelium discoideum is a useful host for the production of constructs for the analysis of structure–function relationships of myosin. Here we describe the use of myosin II constructs containing a single tryptophan residue, at different locations, for probing events at the nucleotide binding site, the relay loop and the communication path between them. GFP fusions have also been expressed at the N- and C-termini of the myosin motor to provide sensitive probes of the actomyosin dissociation reaction in microscope-based kinetic assays. We report on the fluorescence anisotropy of these constructs in the context of their use as resonance energy transfer probes.

Introduction

The ability to carry out standard molecular genetic procedures and to express milligram quantities of functional myosin constructs has made *Dictyostelium discoideum* (Dd) a particularly useful organism in the field of cell motility (Ruppel and Spudich, 1996; Kessin, 2001). Here we review and extend our recent work on the introduction of site-specific fluorescent probes for characterising the mechanism of Dd myosin II. Labelling a protein with fluorescence probes generally involves one of three routes (i) introduction of a tryptophan residue (and/or removal on native tryptophans) to yield a single tryptophan mutant (ii) introduction of a cysteine residue (and/or removal on native cysteines) for directed covalent modification (iii) construction of a fusion with a green fluorescent protein (or related molecule). In this overview we focus on the first and third approaches.

Tryptophan fluorescence

Tryptophan residues in proteins are usually the dominant source of u.v. absorption and fluorescence emission. Although not an abundant residue, most large proteins contain several tryptophan residues and therefore the spectroscopic properties reflect the sum of several components (e.g. a myosin motor domain typically contains 4–5 residues). Use of molecular genetic methods to substitute native tryptophan residues with phenylalanine or tyrosine residues to leave a single tryptophan residue per protein molecule is a useful approach because (i) the spectroscopic signature arises from a single known location within the protein (ii) any heterogeneity in the signal may be analysed less ambiguously than for multi-tryptophan containing proteins (iii) the relative changes in signal on ligand binding may be larger as the background from non-responsive tryptophans is removed. Needless to say in the context of a spectroscopic probe, it is important that the substitution of native tryptophan residues for other aromatic side chains should not significantly alter the overall properties of the protein. Also care is required in selecting the excitation wavelength and slit width to ensure the signal reflects that from the tryptophan residue. The 30 or more tyrosine residues in the myosin motor domain make a significant collective contribution when excited at 280 nm. We generally find that excitation should be at 295 nm or greater, and often employ the 297 nm Hg line from a mercury–xenon lamp.

Tryptophan fluorescence has long been used as an empirical probe of myosin conformation during ATPase activity (Bagshaw *et al.*, 1972; Werber *et al.*, 1972; Bagshaw *et al.*, 1974). Most myosin species respond to ATP binding and hydrolysis with a modest but usable change in signal amplitude (5–20% change). Before the impact of molecular biological approaches, the best characterised system was that from vertebrate skeletal myosin subfragment 1, where it was established that tryptophan fluorescence was enhanced (denoted by *) on both the nucleotide binding and hydrolysis steps (Bagshaw *et al.*, 1974; Johnson and Taylor, 1978). A general scheme to account for these transitions was proposed 30 years ago.

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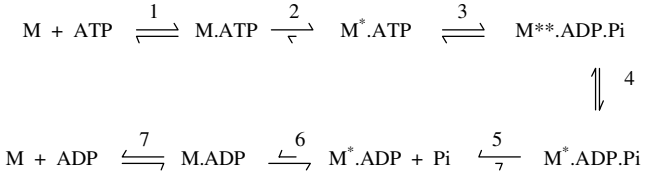
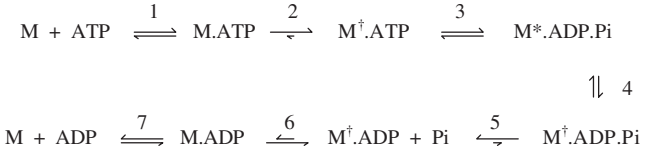


(1)

Interestingly, wild-type Dd myosin lacks tryptophan residues close to the active site (equivalent to W113 and W131 of skeletal muscle myosin) and shows no enhancement in fluorescence on nucleotide binding (in fact there is a small quench denoted with† (Kuhlman and Bagshaw, 1998; Malnasi-Csizmadia *et al*., 2000)). An enhancement in tryptophan fluorescence is observed and has been associated with ATP hydrolysis. Thus the corresponding scheme for Dd myosin II motor is



(2)

Selective chemical labelling studies have enabled particular tryptophans in skeletal muscle myosin to be identified as the major sensors of conformation during the ATPase activity (Park and Burghardt, 2000) but the molecular biological approach has proven more flexible. For example, the latter as shown unambiguously that the conserved relay loop tryptophan (W501 in Dd myosin II) is the sensor of the 'hydrolysis' step 3 in Dd myosin II (Batra and Manstein, 1999; Malnasi-Csizmadia *et al*., 2000) and vertebrate smooth muscle myosin (Onishi *et al*., 2000; Yengo *et al*., 2000). Furthermore, time-resolved studies have shown that the hydrolysis step is a composite one in which a loosely coupled conformational transition (probably corresponding to the open–closed transition identified by crystallography; (Geeves and Holmes, 1999)) precedes the chemical step and that the tryptophan residue actually responds to the former (Malnasi-Csizmadia *et al*., 2001b; Urbanke and Wray, 2001). The detailed kinetic arguments for these conclusions are given in the original papers (Malnasi-Csizmadia *et al*., 2000; 2001a,b; Kovacs *et al*., 2002). Rather, here, we put the information in context and highlight some salient features.

The open–closed transition

While there have been a number of experimental approaches that have indicated that the myosin motor domain can bend (Highsmith and Eden, 1990; Tokunaga *et al*., 1991), the crystallisation of the myosin head in initially two (now three) distinct conformations provided, perhaps, the most convincing and certainly the most detailed description of the sub-domains involved in the movement (Geeves and Holmes, 1999). The original structures of Dd myosin in the ADP.BeF_x and ADP.AlF_4 complexes provided the first clues that, upon 'hydrolysis', the movement of the switch II region was transmitted towards the C-terminus via the relay helix and allowed the converter domain to roll around the latter and could impart a significant rotational motion to so-called lever arm (Fisher *et al*., 1995). Subsequent studies of smooth muscle and scallop myosin crystal structures, with an intact light chain(s) supported and extended these ideas (Dominguez *et al*., 1998; Houdusse *et al*., 2000). In general, it was found that non-hydrolysable nucleotides favoured the 'open' state in which the switch II region was 0.8 nm from the γ-phosphate (or equivalent moiety) and the lever arm was extended, while analogues of hydrolysed nucleotide state (ADP.AlF_4 and ADP.Vi) favoured the 'closed' state and caused the lever arm to swing through around 70°. Solution studies, with probes designed to sense the lever arm position (Suzuki *et al*., 1998; Shih *et al*., 2000), support the idea that these movements are not crystal artifacts, although relating these spectroscopic signals to the crystal structures remains a challenging task.

These structural studies provide a ready explanation as to why the conserved tryptophan (Dd W501) in the relay loop is sensitive to ATP hydrolysis. Why W501 gives a large *enhancement* in fluorescence (70–100% increase in the single tryptophan mutant) is less easy to explain in detail, although a number of contributory factors can be identified. Comparison of the crystal structures for the ADP.Vi complex (pdb:1VOM, (Smith and Rayment, 1996) and ADP.BeF_x (pdb:1G8X, (Kliche *et al*., 2001) suggests the tryptophan becomes slightly less solvent accessible in the closed state, although in other structures (pdb: 1MMD, 1MND, (Fisher *et al*., 1995) the W501 tryptophan is not resolved, indicating substantial disorder. Collisional quenching measurements with acrylamide indicate that W501 is more protected during steady-state ATP hydrolysis (i.e. predominantly the M.ADP.Pi state) than in the M.ADP state, but the difference is small and less than the protecting effect of nucleotide binding to the apo state (Malnasi-Csizmadia *et al*., 2000). Also, it should be stressed that, prior to the enhancement in emission intensity on 'hydrolysis' i.e. the open–closed transition, there is a quench in W501 tryptophan fluorescence associated with the nucleotide binding step. Thus the relay loop is sensing events initiated at the active site earlier in time than the major open–closed transition. Interestingly, comparable studies on engineered smooth muscle constructs indicate that the equivalent W512 tryptophan responds to nucleotide binding with an enhancement, followed by a further enhancement on 'hydrolysis' (Yengo *et al*., 2000). Most likely these changes reflect a common movement of the relay loop, but the different residues around the conserved tryptophan result in fluorescence changes of

different sign and magnitude. The complexity of the observed net emission intensity is also apparent from fluorescence lifetime studies. In common with practically every other single tryptophan containing protein examined (Lakowicz, 1999), the fluorescence lifetime of W501 in all myosin nucleotide states is at least biphasic (Malnasi-Csizmadia *et al.*, 2001a). Together with the existence of a statically quench component, these findings argue that at least three different local conformational states of the tryptophan are present for each 'single' bulk conformation defined for each biochemical state. These microstates interconvert slowly on the nanosecond time scale but rapidly on the millisecond timescale. How these microstates redistribute to give a net enhancement or quench is difficult to predict. It is likely these local conformation states reflect the different rotamers of the tryptophan residue, as well as different rotamers of residues in contact with this side chain.

Interestingly different rotamers for W501 were observed by crystallography in the two motor domains of each unit cell of the Dd myosin motor-α actinin fusion construct (M761-2R-R238E, pdb 1G8X, (Kliche *et al.*, 2001). While it might be possible to pin down a dominant interaction between the tryptophan residues and another side chain (or indeed backbone carbonyl group) that is responsible for the enhancement (or dequench) (Park and Burghardt, 2002), interpreting the amplitude of the fluorescence changes, even of single tryptophan mutants, at present remains semi-empirical. Although these findings might be regarded as an unnecessary complication of the picture, with little relevance to physiological timescales and events, it provides an appropriate reminder as to the dynamics of side chains and care required in interpreting interactions between residues based on small differences in static separation.

How can tryptophan fluorescence studies be used to complement structural studies? The main strength of the former comes from its application as a signal for transient kinetic studies. Here we have shown that the W501 enhancement occurs with a maximum observed rate constant of 30 s^{-1} at saturating [ATP] in stopped-flow experiments, in line with the wild-type protein (Malnasi-Csizmadia *et al.*, 2000). This was generally assumed to provide a measure of the effective hydrolysis step. In fact, W501 senses a coupled reaction, as alluded to above, with an unfavourable but rapid ($\geq$1000 s^{-1}) conformational transition followed by hydrolysis itself, that has an intrinsically higher rate constant than 30 s^{-1}. These processes can only be resolved by perturbation methods (Malnasi-Csizmadia *et al.*, 2001b), because in stopped-flow mixing methods the rapid open–closed transition is limited by a prior isomerisation associated with the binding step. The identification of the observed conformational change as being the open–closed transition rests on the expected change in environment of W501 and the idea that switch II region has to move to bring the catalytic residues in close contact with the γ-phosphate for hydrolysis (i.e. this step must precede hydrolysis (Geeves and Holmes, 1999) – although whether the steps are kinetically resolvable depends on fortuitous values of the rate constants). The scheme shown in Equation (2) was therefore expanded to separate the open–closed transition from hydrolysis.

$$\begin{array}{ccccccccc} \mathrm{M} + \mathrm{ATP} & \overset{1}{\rightleftharpoons} & \mathrm{M.ATP} & \overset{2}{\rightleftharpoons} & \mathrm{M^{\dagger}.ATP} & \overset{3a}{\rightleftharpoons} & \mathrm{M^{*}.ATP} & \overset{3b}{\rightleftharpoons} & \mathrm{M^{*}.ADP.Pi} \\ & & & & & & & & \Updownarrow 4 \\ \mathrm{M} + \mathrm{ADP} & \overset{7}{\rightleftharpoons} & \mathrm{M.ADP} & \overset{6}{\rightleftharpoons} & \mathrm{M^{\dagger}.ADP + Pi} & \overset{5}{\rightleftharpoons} & \mathrm{M^{\dagger}.ADP.Pi} & & \end{array} \qquad (3)$$

The fact that the combined open–closed transition and hydrolysis steps are perturbed by temperature and pressure jumps emphasises another important property – these steps are freely reversible, but favour hydrolysis (i.e. M*.ADP.Pi) overall. This idea (for the overall hydrolysis reaction) was proposed 30 years ago based on quenched flow and isotope exchange studies (Bagshaw and Trentham, 1973; Bagshaw *et al.*, 1975). The significance of this finding is that little energy is wasted in moving the detached crossbridge into a state ready for the next crossbridge cycle (but the cocking of the crossbridge depicted in some text book models is perhaps overly simplistic). The novel recent finding, based in part on the W501 data (Malnasi-Csizmadia *et al.*, 2001b), is that the detached crossbridge can undergo rapid motions, more than an order of magnitude faster than the observed hydrolysis rate, to explore actin binding sites. It is possible that this equilibrium also exists with weakly attached crossbridges because the rate constant for the ATP-induced dissociation of acto-Dd myosin motor (150 s^{-1}) is several times slower than k_{-3a} (Kuhlman and Bagshaw, 1998). However actin may modulate this rate constant, and in a structured system, the open–closed transition of the actin-bound head may be restrained by mechanical coupling. The concept of dynamically disordered weakly attached crossbridges was previously deduced from epr measurements (Thomas *et al.*, 1995).

The relative insensitivity of W501 to the initial nucleotide binding events allows the equilibrium or steady-state fluorescence intensity to provide a measure of the equilibrium constant for the open–closed transition (in combination with the subsequent hydrolysis equilibrium where applicable). In this procedure we assume that the fluorescence emission of the $M^{\dagger}$.ADP and M*.ADP.AlF_4 species represent the fully open and fully closed state respectively. We have extended our original studies to explore ligands such as PPi, ITP and GTP (Table 1). The latter nucleotide shows little phosphate burst with other myosin species (White *et al.*, 1993, 1997) yet it results in a marked fluorescence enhancement of W501 indicating the formation of a small but significant amount of the closed state M*.GTP during the steady-state turnover. These estimates require confirmation by perturbation methods. For example, the apo M state shows a fluorescence yield between the

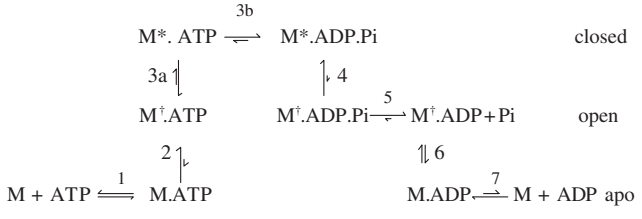
Table 1. Equilibrium constants for the open–closed transition in the presence of different ligands at 20 °C estimated from the fluorescence emission from W501

Ligand	W501 Fluorescence change (%)	$_{app}K_{oc}$	K_{oc}
ADP	−11		<0.05
ATP	+72	≈32	≈0.4
ATPγS	+14	≈0.3	≤0.3
AMP.PNP	+28		≈0.82
ADP.BeF_x	+39		≈1.43
ADP.AlF_4	+72		>20
PPi	−10		<0.02
Pi	<−5		<0.06
ITP	+43	≈1.7	≤1.7
GTP	+25	≈0.7	≤0.7

Tryptophan fluorescence was measured relative to the apo state. These data were collected using several different preparations over a period of 3 years on either the steady-state fluorimeter or stopped-flow apparatus. The data were therefore normalised for each ligand using the observed fluorescence levels in the presence of ADP and ATP as reference points. For hydrolysable nucleotides the observed equilibrium constant ($_{app}K_{oc}$) is affected by coupling to the hydrolysis step.

$M^{\dagger}$.ADP and M*.ADP.AlF_4 values that might suggest it is a equilibrium mixture of open:closed states with K_{eq} of around 0.2. However, pressure jump failed to resolve a transient (Malnasi-Csizmadia *et al.*, 2001b), indicating the equilibrium lies strongly towards one (presumably open) state. In this case, the difference in fluorescence between M and $M^{\dagger}$.ADP is accounted for by a separate isomerisation step (K_6), discussed below. Furthermore, the apo M fluorescence emission peak is red-shifted compared with that of the $M^{\dagger}$.ADP and M*.ADP.AlF_4 complexes, and therefore cannot be in an intermediate conformation.

Consideration of the crystal structures suggests that the product phosphate cannot dissociate from the closed state as the 'back door' is obstructed. One might therefore consider that the M*.ADP.Pi complex must return to the open state before Pi release (Yount *et al.*, 1995). This idea is emphasised by representing the kinetic scheme in terms of three fundamental myosin states; apo, open and closed:



(4)

In order that the M*.ADP.Pi state is the predominant steady-state intermediate, it is necessary for either k_4 to be slow (0.05 s^{-1}) or that K_4 is rapid but ≪1 so that when coupled to the Pi release step, the overall rate constant is slow (i.e. $K_4k_5 = 0.05\ s^{-1}$). These options are difficult to distinguish because the $M^{\dagger}$.ADP.Pi remains at low concentration throughout. Previous attempts to determine K_4 and K_5 as individual equilibrium constants with vertebrate skeletal myosin, via incorporation of Pi into protein-bound ATP, were only part successful because of uncertainties in the effects of ionic strength changes. A model independent estimate of $K_4K_5 =$ 0.19 M was obtained and demonstrated the extremely weak Pi binding to the equivalent of $M^{\dagger}$.ADP (Mannherz *et al.*, 1974; Goody *et al.*, 1977).

Equation (4) implies that the active site must close to allow efficient hydrolysis, but open to allow Pi release. Experimental conditions, ATP analogues, chemical modifications and mutations may favour the open or closed state. Modifications that slightly favour the open state can lead to a slower net hydrolysis rate, but faster Pi release. Given that the latter is the observed rate-limiting step for myosin alone, it is perhaps not surprising that many minor modifications lead to an increase in the basal ATPase rate but reduced actin-activation, because the latter may become limited by the hydrolysis step (Sasaki *et al.*, 2002). More extreme mutations appear to lock the myosin in open (e.g. G457A) or closed (e.g. E459A) states, and severely inhibit both basal and actin-activated ATPase (Sasaki *et al.*, 1998). This effect is also illustrated by mutations that modify the interface between the N-terminal domain and converter region. In the wild type Dd myosin motor there is a charge repulsion between K84 and R704 in the open state, but not in the closed state where the residues are 3.5 nm apart. In mutations such as K84M and R704E where the interaction is neutralised or changed to an attractive force respectively, the level of fluorescence during the steady-state hydrolysis of ATP indicates a higher proportion of the open state (Malnasi-Csizmadia *et al.*, 2002).

Equation (4) indicates how tight binding Pi analogues such as Vi and AlF_4 can drive the formation of an M*.ADP.Pi like state and, in these cases at least, the equilibrium constant equivalent to K_4 is ≪1 for the wild type myosin motor, in order that almost all of the myosin is trapped in a high fluorescent closed state. For BeF_x, K_4 appears to be around 1 at ambient temperatures and thus both open and closed states are nearly equally occupied (Malnasi-Csizmadia *et al.*, 2001b). However, if the Be atom forms a covalent link with the oxygen atom of the β-phosphate of ADP (i.e. it acts as an ATP analog), then the rapid transient observed by relaxation methods might reflect the open–closed transition associated with step 3a. This ambiguity can only be removed using structural methods, such as nmr, to monitor the extent of covalent bond formation (Henry *et al.*, 1993).

The favourable characteristics of the tryptophan emission from the W501+ construct encouraged us to re-examine the formation of the M*.ADP.Pi state by mixing the protein with high concentrations of ADP and Pi. Earlier attempts with vertebrate skeletal myosin failed to detect any enhancement in fluorescence above the skeletal M*.ADP state (Equation (1)). Indeed, a slight reduction in fluorescence was noted and interpreted as

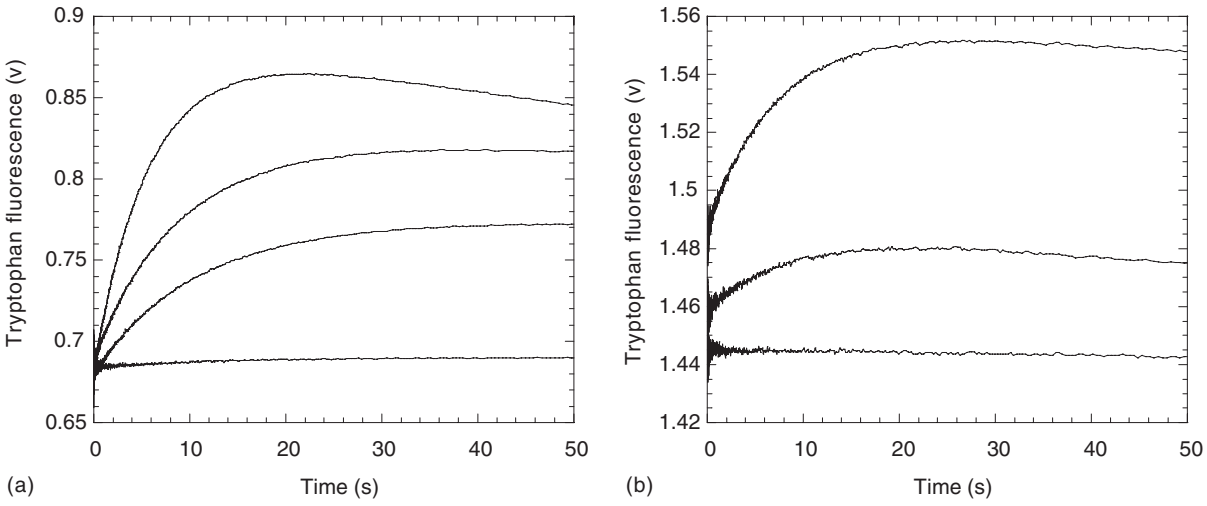


Fig. 1. Formation of ternary complex (presumed M*.ADP.Pi species) on addition of ADP and Pi to W501+. (a) Stopped-flow records obtained on mixing 3 μM W501+ construct plus1 mM ADP with increasing Pi concentrations; 0.25, 5.5, 10 and 30 mM. (b) Stopped-flow records on mixing 3 μM W501+ construct plus 10 mM Pi with increasing ADP concentrations; 0, 0.2 and 0.5 mM. All concentrations refer to final reaction chamber. Note the partial recovery of the signal at high ligand concentrations (see text). Buffer conditions were 40 mM NaCl, 1 mM $MgCl_2$, 20 mM TES at pH 7.0 and 20°C. Tryptophan fluorescence was excited at 295 nm and the emission collected through UG11/WG320 filters.

evidence for a competitive M.Pi species that formed at the expense of M*.ADP (Bagshaw and Trentham, 1974). This complication is less of a problem in Dd myosin because the fluorescence of the corresponding $M^{\dagger}$.ADP is already quenched and addition of Pi to W501+ also caused a 10% quench relative to the apo state (Table 1). Consequently, any enhancement above these levels can be ascribed to ternary complex formation.

Addition of both ADP and Pi to the W501+ construct caused a significant enhancement above the $M^{\dagger}$.ADP and $M^{\dagger}$.Pi levels, suggesting formation of a significant amount of the M*.ADP.Pi species (Figure 1). At 1 mM ADP and 30 mM Pi, the enhancement was about 25% that obtained during the steady-state hydrolysis of ATP, suggesting a K_4K_5 value of around 85 mM. The observed rate constant for the enhancement increased from around 0.1–0.2 s^{-1} with increasing [Pi] and is thus consistent with a reversible step 4 where $k_{-4} > k_4 \geq 0.05\ s^{-1}$. These data are in line with the values observed for the formation of the vertebrate skeletal M*.ATP state (Equation (1)) monitored by the incorporation of ^{32}P from Pi eluded to above (Goody *et al*., 1977). Interestingly, the enhancement observed on mixing $M^{\dagger}$.ADP with Pi or $M^{\dagger}$.Pi with ADP was accompanied by a slow partial reversal. A possible contributory factor is the contaminant PPi present in the Pi stock solution. The data shown in Figure 1 were obtained after the Pi stock solution had been pre-boiled at pH 11 to reduce PPi contamination. Without this step the initial enhancement on adding Pi to $M^{\dagger}$.ADP was much reduced in amplitude as a result of a dominating reversal phase, while no change was detected on adding ADP to $M^{\dagger}$.Pi, presumably due to $M^{\dagger}$.PPi formation.

At pH 6.5 the effect of Pi on the fluorescence enhancement was more marked suggesting about a twofold increase in affinity compared with pH 7.0. Sulphate also appeared to form a ternary M*.ADP.SO_4 complex with about a fivefold higher affinity than Pi. While these effects were observed over the 1–30 mM concentration range, determination of absolute affinities is difficult owing to changes in ionic strength (Goody *et al*., 1977). Nevertheless, they add further support to the concept that the product release steps are relatively reversible and the 'irreversible' step involves the ATP binding isomerisation (Bagshaw and Trentham, 1973; Goody *et al*., 1977).

Nucleotide binding mechanism

The absence of tryptophan residues at the nucleotide binding site of Dd myosin II could account for the lack of a significant fluorescence change on the nucleotide binding compared with skeletal myosin where ADP binding gives around a 7% enhancement (Bagshaw and Trentham, 1974). To test this idea we engineered single trypophan residues into Dd myosin (lacking all native tryptophans) at positions D113 and R131 to mimic, separately, the skeletal myosin residues (Kovacs *et al*., 2002). Although the D113W displayed a small enhancement on nucleotide binding (3%), a dominating contribution would arise from R131W that showed a 30% quench. It is likely that these residues are responsible for the enhancement seen in skeletal myosin on ADP binding, but that the local environment is different due to surrounding non-conserved residues. It is also possible that skeletal W510 (equivalent to Dd W501) also responds with an enhancement on ADP binding (cf. smooth myosin; (Yengo *et al*., 2000), given that in Dd myosin this residue also senses an isomerisation step before the open–closed transition (but gives rise to a 10–15% quench). According to differential quenching

measurements (Park and Burghardt, 2000), skeletal W131 is sensitive to nucleotide binding (16–25% enhancement) but it does not sense subsequent steps, whereas W510 shows an enhancement on both nucleotide binding (38% enhancement on ADP) and the hydroylsis (i.e. open–closed) transition (ATP caused a 95%, and ADP.AlF_4 caused a 102% enhancement relative to the *apo* state). Although our Dd tryptophan mutants did not provide a generally applicable solution as to the contributions from the various tryptophan residues to net fluorescence changes, the R131W construct did provide a useful probe for nucleotide binding without the complications of a signal from the subsequent open–closed transition.

Another mutant F129W provided an even larger signal that R131W and was the focus of detailed characterisation (Kovacs *et al*., 2002). Although the fluorescence signal was in the opposite direction to that observed in skeletal myosin, the Dd F129W construct showed very similar kinetics of nucleotide binding in yielding biphasic kinetic profiles (Trybus and Taylor, 1982). While these ATP binding data could be modelled in terms of two sequential isomerisations (with rate constants of the order of 1800 and 350 s^{-1} for Dd F129W), there was an indication of a third process whereby ATP bound weakly in a non-competent conformation at the active site (Kovacs *et al*., 2002). It is possible that this incorrectly bound substrate can reorient at the active site to join the main pathway. There remains considerable uncertainty as to the nature of these alternative pathways (Tesi *et al*., 1989). As with the W501+ construct, lifetime-resolved fluorescence studies with F129W indicate that each biochemical stable state actually comprises three or more microstates. These measurements were made possible by the increased signal-to-background achieved by engineering a single tryptophan probe as well as improvements in the dead-time of the stopped-flow apparatus. They illustrate a general point that technological advances, besides providing a more precise answer to an existing question, often pose new questions, too. It is likely that protein–ligand interactions and catalysis are more generally described by an energy landscape rather than specific sequential pathway, and thus fitting to discrete exponentials is an approximation. Thus kinetic schemes are a short-hand way of lumping rapidly interconverting intermediates into single states for the convenience of the problem to be addressed while the number of steps resolved reflects the nature of the experiment.

The isomerisations associated with nucleotide binding sensed by F129W are clearly distinct from the open–closed transition sensed by W501, as illustrated in the 'double' mutant (F129W/W501+) where they are clearly resolved (Kovacs *et al*., 2002). The binding isomerisations are more rapid and less reversible than the observed coupled open–closed/hydrolysis transition. We are currently investigating the influence of F-actin on the steps sensed by single tryptophan residues in the myosin motor domain. Interestingly, a fluorescence enhancement occurs when a Dd myosin motor lacking all tryptophans (W−, (Malnasi-Csizmadia *et al*., 2000)) binds to native vertebrate skeletal actin, indicating one or more tryptophan residues in actin is perturbed by this process (cf. (Johnson and Taylor, 1978; Yengo *et al*., 1999)). However the signal-to-noise is sufficient to detect the contributions from single tryptophan residues within the myosin motor, when present, against the background actin signal.

Tryptophan probes in other locations

We have also prepared tryptophan mutants at other locations in the Dd myosin motor domain to probe the path of conformational changes between the nucleotide site and C-terminus. Single tryptophans were introduced into a tryptophan-less background (W−) at the F458 (W458+ construct), F461 (W461+) and F692 (W692+). We also produced a double tryptophan construct having the native W501 and F692W mutation (W501/692+). Based on the atomic structure, the F692 side chain makes a van der Waals interaction with W501, although these residues are located in separate sub-domains (the converter and relay loop, respectively). F458 is a key residue of the switch II loop. F461 is at the start of this loop, at the junction of the actin binding cleft and the relay-converter region.

The F458W mutation caused at least a 20 times reduction in the steady-state ATPase activity and did not show a significant fluorescence emission change on adding nucleotide. We did not characterise it further. In contrast, the W461+ construct had similar steady-state MgATPase activity (0.035 s^{-1}) to the wild-type motor domain (0.03 s^{-1}). The fluorescence emission intensity of W461 was sensitive to nucleotide states, with ADP and ATP showing a 12 and 22% quench in the peak emission at the 345 nm, respectively. The stopped-flow records of the reaction of W461+ with ADP could be fitted to single exponentials and the observed rate constant did not saturate up to 400 μM ADP concentration. These data yielded a second-order rate constant of 2×10^6 M^{-1} s^{-1} and an intercept (dissociation rate constant) value of 10 s^{-1}. This represents a slightly faster binding process than the wild type, however, the equilibrium binding constant is unchanged. On mixing W461+ with ATP below 150 μM nucleotide concentrations, the fluorescence profiles could be fitted by single exponentials but, at higher substrate concentrations, two phases were distinguished. The fast phase represents a second-order binding reaction (rate constant 0.8×10^6 M^{-1} s^{-1}) and did not saturate up to 750 μM ATP concentration, whereas the slow phase reached a maximum of 30 s^{-1} above 200 μM ATP. We suppose that W461 senses both the nucleotide binding and open–closed conformational transition.

The steady-state MgATPase activity was not changed by the F692W mutation. In contrast to the W501+ construct, which shows a fluorescence enhancement of

80% on ATP addition, the fluorescence of the W692+ construct was reduced by 11% compared with the apo form. ADP reduced emission intensity of W692+ by 7%. There was no significant spectral shift with either ATP or ADP. The W501/692+ construct showed a 45% fluorescence enhancement on adding ATP, but the spectra of the apo and ADP forms were indistinguishable. There was no spectral shift on adding nucleotide, however, all three of the spectra (apo, +ADP and +ATP) were blue-shifted (maximum at 333 nm) compared to the apo form of the W501+ construct. While apo W501+ and apo W692+ had similar fluorescence emission intensities, the apo W501/692+ emission was only 67% of their sum at the spectral maxima. To compare the relative self-quenching effect of the nearby tryptophans in the W501+ construct, the areas under the spectra of all three constructs in the presence and absence of ATP and ADP were integrated. We found that in the apo states, the self-quenching effect in the 501/692+ was 3.30 times while in the presence of ATP (or ADP.AlF_4 complex) and ADP it was only 2.60 and 2.99 times, respectively. Given the tight packing of hydrophobic residues in the interface between the relay loop and converter domain, there is little scope for significant distant changes between W501 and W692. However orientation changes are likely, which would affect the efficiency of energy transfer between the tryptophans. Indeed, if the tryptophans reside in different rotamer micro-states, as evidenced by the lifetime and crystallographic results eluded to above, a redistribution of the relative orientations of W501 and W692 side chains in different nucleotide states is a likely outcome.

GFP-motor domain fusions

Green fluorescent protein fusions have proven valuable tools for studying protein function both within cells and *in vitro*. Our incentive for characterising such Dd myosin constructs has been to develop sensitive microscope-based kinetic assays as well as for FRET studies. In particular, it is of interest to follow the paths of conformational changes through the molecule to determine if there is tight coupling between the movement of switch 2, the relay loop and the converter domain. The elegant approach of Suzuki *et al.* (1998) showed a substantial FRET change between a BFP and GFP moiety fused at the N- and C-termini of the Dd myosin II motor domain in different nucleotide states. For quantitative analysis of such data, information on the rotational properties and/or orientation of the fluorophores is required in order to assess the appropriate κ^2 value for the Förster equation. We have initially characterised a single GFP construct fused at the C-terminus of the Dd myosin W501+ motor construct with an intervening Gly–Gly–Gly linker sequence. We have also prepared a YFP fusion attached to the N-terminus of W501+ with no linker. The motor domain was based on the single tryptophan containing W501+ construct. These fusions gave a well-resolved tryptophan peak that responded to ATP binding with kinetics similar to the W501+ construct alone (Figure 2a). As expected, the fluorescence from GFP moiety itself was insensitive to ATP binding and hydrolysis (Figure 2b). Furthermore we could detect no change in energy transfer from tryptophan to GFP on addition of ATP. However it is likely that the energy transfer from Trp to GFP is dominated by the two nearby tryptophans in the GFP moiety (van Thor *et al.*, 2002), and any FRET between W501 and GFP fluorophore is negligible. Steady-state anisotropy measurements on the isolated GFP protein and the myosin motor-fusion indicated that the fluorophore was relatively immobile on the nanoseconds timescale (Table 2). These data are in line with earlier reports (Chattoraj *et al.*, 1996) and are to be

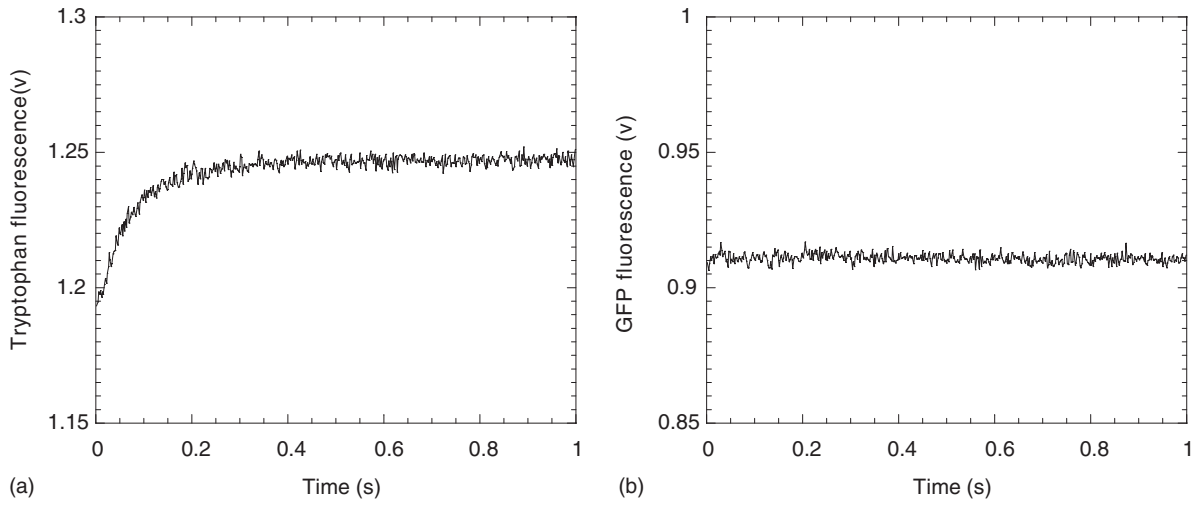


Fig. 2. Stopped-flow traces of ATP interaction with the W501+–GFP construct monitored by (a) tryptophan fluorescence and (b) GFP fluorescence. ATP (25 μM) was mixed with 0.5 μM protein (reaction chamber concentrations) in 40 mM NaCl, 20 mM HEPES and 2 mM $MgCl_2$ at pH 7.5 and 20°C. Tryptophan fluorescence was monitored by excitation at 295 nm and the emission selected with UG11 and WG320 filters. GFP fluorescence was excited at 490 nm and monitored with an OG515 cut-off filter.

Table 2. Fluorescence anisotropy values were measured at 490 nm excitation (fluorescein and GFP) or 510 nm (YFP)

Fluorophore	Ligand	Anisotropy
GFP	–	0.295 ± 0.008
Dd W501+–GFP	–	0.311 ± 0.002
Dd W501+–GFP	F-actin	0.311 ± 0.003
Dd W501+–GFP	ATP	0.311 ± 0.003
YFP–Dd W501+	–	0.311 ± 0.002
Fluorescein	–	0.046 ± 0.002

The instrument gave a value of 0.99 for the scattering signal from dilute glycogen.

expected from the molecular volume of GFP alone. Tethering GFP to the motor domain causes only a slight additional restriction. The anisotropy of the fusion protein was insensitive to ATP addition, both in the steady-state and in polarisation-resolved stopped-flow measurements. The value was also unchanged when the fusion protein was bound to actin filaments. The difference from the theoretical maximum anisotropy value of 0.4, for totally immobilised fluorophores randomly distributed in solution, probably arises from a small difference between the absorption and emission dipoles of the GFP flourophore (Lakowicz, 1999; Boxer and Rosell, 2002). These data indicate that a κ^2 value of 0.67, deduced for pairs of rapidly rotating fluorophores, would be inapplicable to GFP fusions.

Actin filaments, decorated with the Dd myosin motor–GFP fusion, were readily observed under the fluorescence microscope. When the emitted light was resolved into two planes of polarisation, there was no marked difference between the intensity from filaments aligned along or across the plane of polarisation. This observation argues that either the GFP domains are not well ordered, or the preferred direction of the dipoles is neither parallel nor perpendicular to the actin filament axis, but at some intermediate angle that becomes averaged out along the actin helix. These possibilities can be separated at the level of single molecule fluorescence (Forkey *et al.*, 2000).

The visualisation of GFP–myosin motors bound to actin by total internal fluorescence microscopy (TIRF) provides a very sensitive method for determining the kinetics of ATP-induced dissociation. Addition of caged ATP at mM concentrations to the GFP–Dd myosin II motor decorated actin filaments causes little or no loss in signal intensity. However on flash photolysis at 350 nm (Conibear and Bagshaw, 2000), the GFP fusion protein is quickly released from the actin (Figure 3). Control experiments in the absence of caged ATP shows that the flash does not cause significant photobleaching of the GFP moiety. Interestingly, when the same experiments were performed with YFP-fusion proteins, a small enhancement was seen in the control that we interpret as photoactivation of the dark state of YFP (Miyawaki and Tsien, 2000). This phenomenon did not prevent the analysis of the ATP induced dissociation, which was the predominant transient, but it opens up possibilities of local photoactivation as a tool, particularly inside cells.

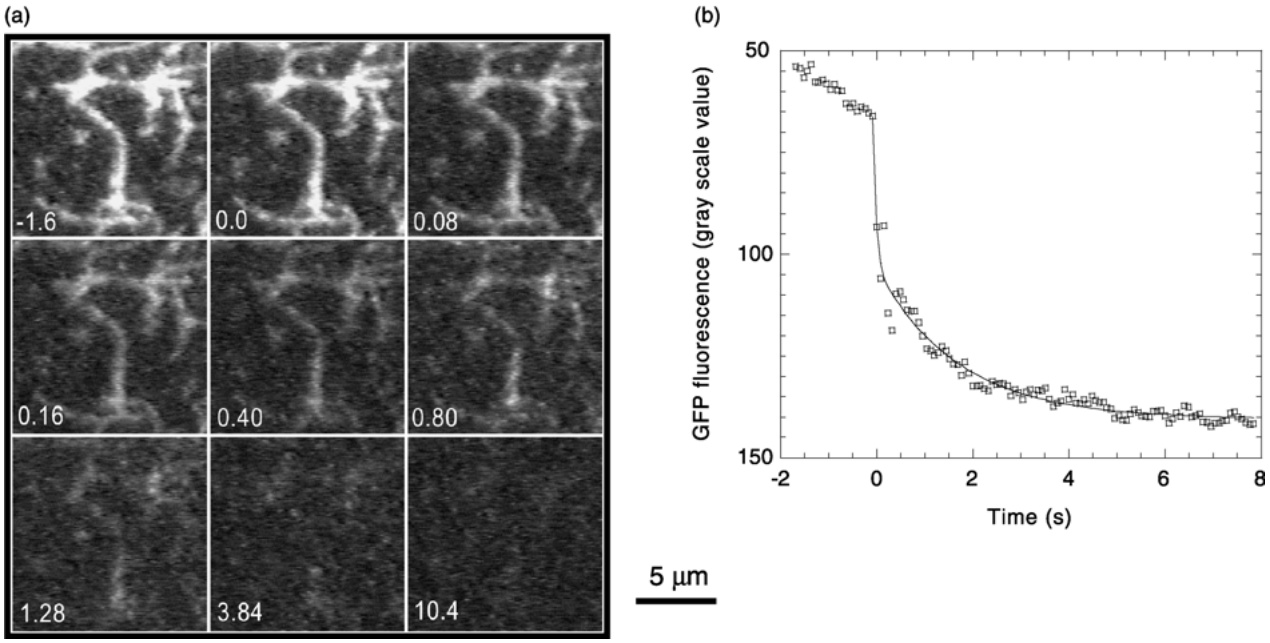


Fig. 3. Dissociation of the W501+–GFP construct from actin filaments on flash photolysis of caged ATP monitored using TIRF microscopy. F-actin filaments were immobilised on a surface of aged rabbit heavy meromyosin which formed a significant number of ATP resistant rigor bonds in a flow cell. The actin was perfused with 40 nM W501+–GFP construct and imaged using TIRF microscopy with excitation at 488 nm with an argon ion laser. Caged ATP (100 μM) in 20 mM KCl, 10 mM MOPS, 5 mM $MgCl_2$, 0.1 mM EGTA, 10 mM DTT at pH 7.2 and 20°C was added and flash photolysis used to release about 20 μM ATP (Conibear and Bagshaw, 1996; Conibear and Bagshaw, 2000). (a) Montage of images taken at time intervals indicated in seconds with the flash at zero time (see movie on CD). (b) Intensity decay of GFP fluorescence measured from the averaged gray-scale value of an individual filament. The record was fit to a biphasic exponential with rate constants (amplitude) ≈15 s^{-1} (0.5) and 0.6 s^{-1} (0.5), the fomer being limited by the video acquisition.

These assays can be performed with a few microlitres of sample at 40 nM stock GFP–myosin fusion protein thereby extending the sensitivity of the flash photolysis approach (Weiss *et al*., 2000). Assays of this type offer scope for combined kinetic and motility characterisation, particularly for processive myosin species that show a lag in dissociation or discernable sliding before release (Rock *et al*., 2001).

Discussion

The introduction of site-specific probes in Dd myosin motor domain has enabled some correlations to be made between structural and kinetic events. In particular the relay loop tryptophan, W501 has allowed the kinetics of the open–closed transition to be resolved from the hydrolysis step. These studies have also confirmed the reversibility of this transition and provide better time resolution that previous isotope exchange measurements. When bound to actin, the reversal of the open–closed transition could provide a mechanism for the crossbridge power stroke. However in this case, the reaction needs to be relatively irreversible, by coupling to Pi release, in order that mechanical work can be done.

W501 also senses the initial binding isomerisation and shows that the apo and ADP bound states are conformationally distinct. This effect has also been observed for the corresponding W512 residue in smooth muscle myosin constructs (Yengo *et al*., 2000) and may be related to the ADP-induced angle change observed in the regulatory domain when bound to actin filaments (Whittaker *et al*., 1995). In the case of ATP binding, there is a considerable free energy drop associated with binding isomerisation (step 2) that is used, in effect, to drive the dissociation of actomyosin (i.e. the so-called R to A transition). In principle, this energy could be coupled directly to the crossbridge power stroke. Thus it is possible in the two-step binding of the myosin motor to actin, there is an inherent conformational transition (e.g. change in angle of motor attachment) associated with the A to R transition leading to tight binding (Geeves *et al*., 1984). ATP binding must reverse this step, however, the A.M.ATP intermediate could be so short lived that the negative tension developed is insignificant relative to that developed over the whole cycle (Eisenberg and Greene, 1980). This idea has recently been tested by Sleep and coworkers (personal communication) using single motor, optical trap measurements. They find there is no inherent stroke associated with tight actin binding to myosin (i.e. rigor bond formation), but rather they suggest the stroke depends on the motor spending a significant proportion of its lifetime in the closed state when detached i.e. only binding via the closed state leads to significant net displacement of the actin filament. The fluorescence levels reported by W501 (Table 1) therefore provide a useful means of testing these ideas with different analogs. The open–closed state equilibrium could be modulated in different myosin isoforms to change the duty ratio, as suggested by the K84M and R704E mutants that probe the interface between the N-terminal domain and the converter region. The K84 residue is conserved in myosin II isoforms but not other members of the myosin superfamily.

Changes in tryptophan fluorescence emission intensity remain an empirical probe that is difficult to relate to the extent of the associated structural change. FRET probes provide a more direct structural approach but, in the case of GFP fusions, their large size prevents rapid rotation of the fluorophore and hence the orientation factor remains undefined. On the other hand, small probes, introduced by covalent modification of engineered cysteine residues, are difficult to label as stoichiometric FRET pairs within a single polypeptide chain. A combination of a single GFP fusion with an engineered cysteine residue may provide a compromise solution. Alternatively, pairs of cysteine residues can be labelled with a single probe that that is sensitive to separation distance and orientation, such as nitroxide spin labels or pyrene eximers (Malnasi-Csizmadia *et al*., in press).

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