Analysis of Nucleotide Binding to Dictyostelium Myosin II Motor Domains Containing a Single Tryptophan Near the Active Site*

Received for publication, March 6, 2002, and in revised form, April 17, 2002
Published, JBC Papers in Press, April 23, 2002, DOI 10.1074/jbc.M202180200

Mihály Kovács‡§, András Málnási-Csizmadia‡§, Robert J. Woolley‡, and Clive R. Bagshaw‡
From the ‡Department of Biochemistry, University of Leicester, Leicester LE1 7RH, United Kingdom and the §Department of Biochemistry, Eötvös Loránd University, Pázmány Péter Sétány 1/C, H-1117 Budapest, Hungary

Dictyostelium myosin II motor domain constructs containing a single tryptophan residue near the active sites were prepared in order to characterize the process of nucleotide binding. Tryptophan was introduced at positions 113 and 131, which correspond to those naturally present in vertebrate skeletal muscle myosin, as well as position 129 that is also close to the adenine binding site. Nucleotide (ATP and ADP) binding was accompanied by a large quench in protein fluorescence in the case of the tryptophans at 129 and 131 but a small enhancement for that at 113. None of these residues was sensitive to the subsequent open-closed transition that is coupled to hydrolysis (i.e. ADP and ATP induced similar fluorescence changes). The kinetics of the fluorescence change with the P129W mutant revealed at least a three-step nucleotide binding mechanism, together with formation of a weakly competitive off-line intermediate that may represent a nonproductive mode of nucleotide binding. Overall, we conclude that the local and global conformational changes in myosin IIs induced by nucleotide binding are similar in myosins from different species, but the sign and magnitude of the tryptophan fluorescence changes reflect nonconserved residues in the immediate vicinity of each tryptophan. The nucleotide binding process is at least three-step, involving conformational changes that are quite distinct from the open-closed transition sensed by the tryptophan Trp501 in the relay loop.

Interaction of nucleotides with vertebrate skeletal muscle myosin is accompanied by an enhancement in tryptophan fluorescence that has long been exploited to determine the mechanism of nucleotide binding and hydrolysis (1–3). Non-hydrolyzable nucleotides (e.g. ADP) cause a smaller enhancement than hydrolyzable ones (e.g. ATP), indicating that part of the fluorescence change observed with ATP may be associated with the hydrolysis step itself. The kinetics of binding saturate at high nucleotide concentrations, indicating that the process is at least two-step (1). Under some conditions, the fluorescence change associated with hydrolysis was resolved from a faster isomerization step (2). A more precise mechanism (Reaction 1) for the fluorescence enhancement associated with hydrolysis has recently been proposed using

* This work was supported by the BBSRC, Wellcome Trust, and the Magyary Zoltán Fellowship. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by EMBO and the Hungarian Eötvös State Fellowship.
§ To whom correspondence should be addressed. Tel.: 44-116-252-3454; Fax: 44-116-252-3369; E-mail: crb5@le.ac.uk.

This paper is available on line at http://www.jbc.org
Nucleotide Binding to Myosin Mutants

M + ATP → M·ATP → M²ATP → M³ATP → M³ATP·P

M + ADP → M·ADP → M²ADP·Fi → M³ATP·Fi

REACTION 1

serveable in the wild-type D. discoideum motor domain (8, 9). These studies showed the nucleotide binding process is at least three-step, involving conformational changes that are quite distinct from the closed-transition. Part of this work has previously been reported (10).

EXPERIMENTAL PROCEDURES

Nucleotides—ATP (special quality, vanadate-free) was from Roche Molecular Biologicals. Other nucleotides were purchased from Sigma. The MADP-BeF₃ complex (where BeF₃ represents beryllium fluoride with undefined stoichiometry) was prepared by incubation of 5–20 μM motor domain, 50 μM ATP, 3 mM NaF, 50 μM BeCl₂ for 30 min. The M·ADP·BeF₃ complex was made similarly but with 50 μM AlCl₃, and incubation for at least 2 h. MantATP² was purchased from Molecular Probes, Inc. (Eugene, OR).

Protein Engineering and Preparation—The constructs used in this study were based on the D. discoideum M761 motor domain (8) and thus have a modified N terminus, MTDXEIP... in place of MNP... and a His-tagged C terminus... RLGSTRDALH 8, where Arg761 is the last residue in the wild-type D. discoideum motor domain (8, 9). In the W113 F129W, and W131 W129 and R131W, respectively. The upstream primer used was 5'-ATAGGTGCTCCTCTGAATGATTGGACACCCAAAAGCAGTCATAGCTG...GTAATAAATACCTAATGTTAGGGACAACCGG-3' for D113W, 5'-TGG-AATTTCTCTCTTCACTGATGTTGAGCCGACCAACCCAGC-3' for D113W, 5'-TGG-AATTTCTCTCTTTCACTGATGTTGAGCCGACCAACCCAGC-3' for D113W, 5'-ATCA...ACATCTTCTTTCACTGTAGTGAATGTTGATGCACTTCTTTACCTTGATGTTGAGCCGACCAACCCAGC-3' for F129W, and 5'-ATCA...ACATCTTCTTTCACTGTAGTGAATGTTGATGCACTTCTTTACCTTGATGTTGAGCCGACCAACCCAGC-3' for R131W as the mutagenizing primer. A similar mutagenizing procedure was performed as in the case of W129 to get the W129 l and 20-μM cell was 0.5 ms, and thus, considering the 3-fold reduction in dead time, the smaller cell provided better resolution of fast (500 s⁻¹) processes. ADP dissociation from the motor domain constructs was followed by rapid mixing with excess mant-ATP and monitoring mant fluorescence (excited at 356 nm) through a 540 filter. Samples containing 2 μM protein and 50 μM ADP were mixed with mant-ATP solutions at varying concentrations and 100 μM ADP dissociation was measured using time-resolved fluorescence measurements. All measurements were performed under buffer conditions 40 mM NaCl, 20 mM TES, 1 mM MgCl₂ at pH 7.5 in a thermostated cell. The reagent concentrations stated are reaction chamber concentrations. Data were collected on a logarithmic time base to ensure that there were a significant number of data points for all phases of multistep reactions (15). The ram profile of the instrument was checked by monitoring its position using the in-built linear potentiometer. Reaction profiles were analyzed by fitting to exponential functions using Origin, version 6.0 (Microcal Software). Reaction pathways were simulated using KinTek-Sim software (KinTek Corp.; www.kintek-corp.com/).
tryptophan represents a conservative substitution in close proximity to the adenine moiety of the bound nucleotide (17, 18) (Fig. 1, a and b). The latter position is occupied by an aromatic residue in almost all myosin classes sequenced to date (19). Thus, with these mutants the spectroscopic changes coming from Trp$^{113}$ or Trp$^{131}$ of skeletal muscle myosin might be mimicked in the absence of the background signal arising from the other tryptophans. The W129$^{	ext{+}}$/W501$^{	ext{+}}$ (W36F, W432F, W584F, and F129W) double tryptophan mutant also contains the conservative Trp$^{501}$ residue (Trp$^{510}$ in skeletal myosin, Trp$^{512}$ in smooth muscle myosin) that has been identified as the residue responsible for the fluorescence enhancement concomitant with the open-closed conformational transition coupled to the hydrolysis step (4, 5, 20–23).

All mutants showed high levels of expression, and ~4 mg of recombinant protein could be prepared per liter of D. discoideum culture, whereas in the case of W129$^{	ext{+}}$ the yield was around 15 mg/liter. With the highly efficient affinity purification procedure, protein preparations of >99% purity could be obtained as shown in Fig. 2. Within experimental accuracy, the basal steady-state MgATPase activity of W129$/W501^+$ was the same as that of the wild type D. discoideum motor domain (0.047 ± 0.06 s$^{-1}$). In the case of W129$^+$ and W131$^+$, the rate was slightly altered by the mutations introduced (0.036 ± 0.04 s$^{-1}$ and 0.080 ± 0.10 s$^{-1}$, respectively). W113$^+$ yielded a rate of 0.05 s$^{-1}$ (single measurement). These findings are in line with previously reported observations that mutating the Trp$^{501}$ residue to phenylalanine at most only moderately affects the ATPase rate (4), whereas in the W501Y-2R construct containing an artificial lever arm, the basal ATPase was not altered (20).

Steady-state Fluorescent Properties—Fig. 3a shows the uncorrected fluorescence emission spectra of the W113$^+$, W129$^+$, and W131$^+$ mutants in the absence of nucleotide and in the presence of ADP. The excitation wavelength was 297 nm with narrow slits (1-nm bandwidth) to obtain maximal intensity changes on adding nucleotide. Both W129$^+$ and W131$^+$ responded with a large quench in fluorescence (55 and 30%, respectively) on ADP binding that was accompanied by a 7-nm red shift in both cases. ADP binding to W113$^+$ caused a small (3%) fluorescence increase and a 2-nm red shift in the emission. Adding ATP or nucleotide analogs to these constructs had the same effect on the emission spectra, which implies that none of these side chains are sensitive to the open-closed transition that follows step 2 (Reaction 1). The increase in intensity when ATP was added to W113$^+$ was slightly higher than that for ADP (~5%, data not shown), but the difference between the signal of the ADP-bound state and the predominant component during steady-state ATPase was not large enough to clearly distinguish the different nucleotide-bound states. Interestingly, the binding of pyrophosphate to the W129$^+$ motor domain causes a large increase (60%) and a 3-nm red shift in the fluorescence signal (Fig. 3b). As shown by crystallographic studies (24), pyrophosphate binds into the β- and γ-phosphate sites of the myosin active site. These data suggest that the decrease in W129$^+$ fluorescence on binding of nucleotide may have a contribution from a direct quenching effect of the adenine moiety.

The addition of ATP to the W129$/W501^+$ construct caused a 50% increase in fluorescence and a 6-nm blue shift of the spectrum during the steady-state reaction, whereas a 30% quench and a smaller blue shift was seen on ADP binding (data not shown). These data, together with the finding that the $I_{W129^+}/I_{W501^+}$ signal intensity ratio in the apo state is 0.56, are consistent with the 55% quench of W129$^+$ emission upon nucleotide binding and a +80 to +100% and −17% signal change seen in W501$^+$ upon adding ATP and ADP, respectively (4). The additivity of these signals might be expected because these side chains are 3 nm apart in the D. discoideum motor domain crystal structures (18) and thus unlikely to interact through efficient energy transfer ( Förster distance = 0.9–1.8 nm) (13).

Dynamics of the Components of the W129$^+$ Fluorescence Emission—The good protein yield of the W129$^+$ construct, the large fluorescence change upon nucleotide addition, and its insensitivity to other events (i.e. open-closed transition) make it the most suitable mutant for analysis of the nucleotide binding site; therefore, we carried out more extensive measurements on this construct. Acrylamide quenching and time-resolved fluorescence measurements were performed to assess the origin of the signal changes. Upon the addition of nucleotide, $K_{quv}$ drops to approximately half of the original value (Table I), which indicates that the Trp$^{129}$ side chain is more shielded from the solvent when ATP or ADP is bound in the active site. The decrease in the W129$^+$ fluorescence quantum yield upon nucleotide binding largely reflects the quench in fluorescence emission intensity (Table I), since its absorbance does not change significantly.
Fluorescence intensity decay times were measured by time-resolved single photon counting. The decay profile was biphasic with 1- and 5-ns phases in all states (Fig. 4). Biphasic decay profiles with similar lifetime components have previously been observed with the W501 single tryptophan construct (16).

From these profiles and the observed quantum yields (assuming an intrinsic lifetime of 16 ns for free tryptophan), the proportion of the third, statically quenched component was calculated. Upon interaction with nucleotide, the proportion of the static component increases, and the relative proportion of the 5- and 1-ns components also changes from about 0.55–0.45 in apo to 0.45–0.55 in nucleotide-bound states (Table I). The amplitude of the decrease in the steady-state fluorescence intensity upon nucleotide addition can largely be accounted for by the increase in the proportion of the statically quenched tryptophan. This suggests that the quench could be due to the direct interaction of the indole moiety with the adenine ring, which is known to be a static quencher.

The high steady-state anisotropy of W129 (about 0.2) shows that this side chain is rather immobile. In the nucleotide-bound states, slightly higher values were observed compared with the nucleotide-free state. Fluorescence anisotropy decay profiles were fitted to single exponentials to yield time constants above 100 ns (Table I). The values are in the time range of the rotational correlation time expected for the whole protein molecule and therefore indicate that the Trp129 side chain is immobile in all states. Similar measurements with W501 gave decay times around 50 ns (16), suggesting that the emission dipole of the latter side chain is aligned along the short axis of the motor domain, whereas Trp129 is positioned along the long axis. Trp131 is also located close to the adenine moiety of the bound nucleotide and shows similar characteristics to Trp129 (i.e., the decrease in fluorescence quantum yield on ATP or ADP binding reflects mainly the change in emission intensity, and this side chain also has a high steady-state anisotropy with a further small increase on nucleotide binding) (Table I).

### Table I

|                     | W129+ |          |          | W131+ |          |          |
|---------------------|-------|----------|----------|-------|----------|----------|
|                     | apo   | ATP      | ADP      | apo   | ATP      | ADP      |
| Quantum yield       | 0.056 | 0.021    | 0.026    | 0.029 | 0.016    | 0.018    |
| Steady state anisotropy | 0.20 | 0.21    | 0.21    | 0.18  | 0.20    | 0.20    |
| $K_{sv}$ (acrylamide, M⁻¹) | 3.86 | 1.80    | 1.52    |       |         |         |
| $t_1$ (ns)          | 5.00  | 4.74    | 4.66    |       |         |         |
| $t_2$ (ns)          | 0.93  | 0.75    | 0.71    |       |         |         |
| Fluorescence lifetime components (%) | Static | 70 | 86 | 84 |       |         |         |
|                     | $t_1$ | 16       | 6        | 7     |         |         |
|                     | $t_2$ | 14       | 8        | 9     |         |         |
| Limiting anisotropy in time-resolved measurement | 0.167 | 0.205 | 0.191 |       |         |         |
| Anisotropy decay time (ns) | 120 ± 9 | 169 ± 24 | 118 ± 14 |       |         |         |

**Fig. 3.** a, uncorrected fluorescence emission spectra of the single tryptophan mutants W113+, W129+, and W131+ in nucleotide-free (solid line) and ADP-bound (dashed line) states. b, emission spectra of the W129+ motor domain in the absence of nucleotide and in ATP, ADP, and magnesium pyrophosphate. Spectra of 10 μM protein samples in 20 mM TES, 40 mM NaCl, 1 mM MgCl₂ at pH 7.5 were recorded at 20 °C (excitation wavelength was 297 nm, with 1-nm band widths). Added ligand concentrations were 1 mM ATP, 200 μM ADP, or 1 mM PP₃.

**Fig. 4.** Intensity decay profile of the W129+ fluorescence emission measured by time-resolved single photon counting. The decay was biphasic with ~1- and ~5-ns phases in all states (Fig. 4). Biphassic decay profiles with similar lifetime components have previously been observed with the W501+ single tryptophan construct (16). From these profiles and the observed quantum yields (assuming an intrinsic lifetime of 16 ns for free tryptophan), the proportion of the third, statically quenched component was calculated. Upon interaction with nucleotide, the proportion of the static component increases, and the relative proportion of the 5- and 1-ns components also changes from about 0.55–0.45 in apo to 0.45–0.55 in nucleotide-bound states (Table I). The amplitude of the decrease in the steady-state fluorescence intensity upon nucleotide addition can largely be accounted for by the increase in the proportion of the statically quenched tryptophan. This suggests that the quench could be due to the direct interaction of the indole moiety with the adenine ring, which is known to be a static quencher.

The high steady-state anisotropy of W129 (about 0.2) shows that this side chain is rather immobile. In the nucleotide-bound states, slightly higher values were observed compared with the nucleotide-free state. Fluorescence anisotropy decay profiles were fitted to single exponentials to yield time constants above 100 ns (Table I). The values are in the time range of the rotational correlation time expected for the whole protein molecule and therefore indicate that the Trp129 side chain is immobile in all states. Similar measurements with W501 gave decay times around 50 ns (16), suggesting that the emission dipole of the latter side chain is aligned along the short axis of the motor domain, whereas Trp129 is positioned along the long axis. Trp131 is also located close to the adenine moiety of the bound nucleotide and shows similar characteristics to Trp129 (i.e., the decrease in fluorescence quantum yield on ATP or ADP binding reflects mainly the change in emission intensity, and this side chain also has a high steady-state anisotropy with a further small increase on nucleotide binding) (Table I).
Nucleotide Binding to Myosin Mutants

Transient Kinetics of Nucleotide Binding—The large signal changes due to the incorporation of an intrinsic fluorophore into the binding site allowed better resolution of the myosin nucleotide-binding transients than was possible in previous studies on skeletal muscle myosin. Also, to follow the very rapid processes in binding (around 1000 s⁻¹), we replaced the original 20-μl flow cell of the SX18MV stopped-flow apparatus with a 5-μl cell to obtain submillisecond dead time. The data were recorded on the same log time base throughout to avoid the effect of switching linear time bases on the weighting of the fits (15). Fig. 5 shows representative fluorescence stopped-flow traces of ADP binding to the W129+ motor domain. The observed quench is of similar amplitude to that seen in the emission spectra. When the curves were fitted to a single exponential, the observed rate constant showed saturation at high [ADP] with a maximum rate constant of about 1300 s⁻¹ and an initial slope of 1.2 × 10⁶ M⁻¹ s⁻¹. However, the data at intermediate ADP concentrations (100–400 μM ADP) showed clear deviations from the fit, indicative of at least a biphasic process. The data were therefore analyzed using a double exponential, which gave a marked improvement in the residues at intermediate [ADP], whereas the improvement in fit was marginal at the highest and lowest [ADP] used. A plot of the kobs of the fast and slow phases against [ADP] yielded apparently distinct profiles (Fig. 6, a and b). The fast phase appeared to yield a large intercept value (kobs ~ 500 s⁻¹), but its precise value was uncertain because at low [ADP] the amplitude was small. The slow phase had an intercept value of <5 s⁻¹ in line with the expected dissociation rate constant (see below). At high [ADP], the fast and slow phases appeared to saturate at about 1000 s⁻¹ and 150 s⁻¹, respectively, at 20 °C, but the amplitude of the slow phase was only about 20% of the total change (Fig. 6c). The high intercept value and shallow slope of the fast phase suggest the presence of a binding step with a low affinity (K > 500 μM). However, a simple in-line mechanism could not be found that would satisfy both a large intercept value and the apparent Kf of the slow phase of around 100 μM. Rather, two separate but competitive association processes were required. A number of schemes were modeled with this feature, including the Trybus-Taylor (3) mechanism with an extra weakly competitive nonproductive binding step to yield Mg⁺-ADP (Reaction 2). Overall, this side branch has little effect on the flux through the pathway, but it was sufficient to account for a positive intercept in the fast kobs.

Simulations of Reaction 2 showed that the resultant profiles were triphasic, but only at intermediate [ADP] were three processes resolvable, and even then, the fit of a triphasic exponential was only a marginal improvement over a biphasic fit. When a simulated set of curves was analyzed by force fitting a biphasic exponential across the concentration range, a rate constant profile close to the experimental data could be generated (Fig. 6). The amplitude data also were reproduced by this scheme.

A second complication of the analysis arises from measuring rate constants in which a significant proportion of the reaction is lost in the dead time of the stopped-flow apparatus. The 5-μl cell has a dead time of 0.5 ms, and thus 40% of the amplitude of a reaction at 1000 s⁻¹ is lost. The fluorescence profiles were analyzed in two ways to check the effect of the dead time losses. Either the origin of the curve was taken at the estimated time zero of the reaction and the amplitude was calculated from the fit at this time, or the fit was restricted to data points collected after the dead time. Either way, the resultant kobs against [ADP] had similar profiles, but the actual values of the rate constants differed slightly. The fluorescence signal determined by extrapolation to zero time coincided with the fluorescence level determined by mixing the W129+ construct against buffer. Thus, these approaches were reasonably consistent and indicated that the loss in signal amplitude was more or less than expected on the basis of the resolved phases of the reaction and the known dead time. Thus, the total amplitude of the signal after correction for dead time was practically constant across the range of ADP concentrations above the overall Kd. When fitted to a biphasic curve, the corrected absolute amplitude of the fast phase increased from a 5 to 40% quench, and the slow phase decreased from a 45 to 5% quench with increasing [ADP]. At 100–200 μM [ADP], the contributions of the phases were roughly equal, but from the argument above, this apparent Kd is partly a reflection of fitting a biphasic curve to a triphasic profile. The same general results were found for the ADP binding profiles measured at 5 °C. The “fast phase” was not particularly temperature dependent, whereas kobs of the slow phase decreased nearly 2-fold.

ATP binding to W129+ showed essentially similar profile to that of ADP. The amplitudes of the profiles were practically identical, indicating that there is little or no contribution of the open-closed transition or hydrolysis step to the signal from Trp129. The maximum rate of the slow phase was 350 s⁻¹ at 20 °C, and the intercept value of the fast phase was possibly slightly higher (<1100 s⁻¹) and saturated at 1800 s⁻¹.

Similar fluorescence profiles were obtained for the W131+ construct with ADP and ATP, but the overall amplitude was reduced to about 25% so that the extraction of phases was not as reliable. On the other hand, the enhancement seen for ADP and ATP binding to Trp131 was too small to yield reliable fits to multiphasic exponentials. A single exponential fit yielded similar rate constants to those observed when the W129+ and W131+ data were force-fit to a single exponential at low concentrations (apparent kobs = 1.3 × 10⁶ M⁻¹ s⁻¹), whereas loss of amplitude at high [nucleotide] prevented an estimate of the maximum kobs. We also note that the kobs of ADP binding transients of the W501+ mutant also showed very similar concentration dependence, and there were indications that the binding process saturated with a rate constant > 250 s⁻¹ (4). Thus, it is possible that the Trp501 side chain, which is located 3 nm from the binding site, is also sensitive to the events reported by Trp129 and Trp131, but the fast and slow phases were unresolved because of the small total amplitude of the
**FIG. 6.** Rate and amplitude data from biphasic fits of the W129+ ADP binding transients (see Fig. 5), plotted against nucleotide concentration. Traces could be fitted to double exponential functions without major systematic deviations throughout the whole concentration range studied. Rate constants for the fast and slow phases are shown in a and b, respectively. Small solid symbols indicate data points where the fit was less certain because the amplitude was small. c, amplitudes of the fast (solid squares) and slow (open triangles) phases, relative to the total signal level. The solid lines with crosses were obtained from a biphasic fit to simulated data for a three-step scheme (Reaction 2) in which \( k_1 = 10 \mu M^{-1} s^{-1} \), \( k_2 = 1300 s^{-1} \), \( k_{3a} = 500 s^{-1} \), \( k_{3b} = 140 s^{-1} \), \( k_{obs} = 10 s^{-1} \), with a competing branch step \( k_2 = 2 \mu M^{-1} s^{-1} \), \( k_3 = 700 s^{-1} \) (i.e., the overall \( K_j \) for ADP binding was about 1 \( \mu M \)). Simulation of the observed dissociation rate constant of ADP from the M\(^{\#}\)ADP by displacement state gave \( k_{obs} = 2 s^{-1} \), whereas the intercept value of the “slow” phase in the simulated data was 7 \( s^{-1} \). It is apparent that the intercept value of the “fast” phase is dominated by \( k_{obs} \), whereas the maximum rate constant of the “fast” and “slow” phases have dominant contributions from \( k_{3a} \) and \( k_{3b} \), respectively. However, these assignments are not precise because there are three rather than two phases present (see “Results”), and each phase has a significant contribution from several rate constants. The addition of a further step that allowed direct rearrangement of the M\(^{\#}\)L species to M\(^{\#}\)L without dissociation and rebinding had a marginal effect on the transient profile. In the simulation shown, the quench of the M\(^{\#}\)L species was twice that of the M\(^{\#}\)L and M\(^{\#}\)L species. This is the maximum quench that can be assigned to M\(^{\#}\)L, since it corresponds to a total quench relative to the apo state (M).

\[
\begin{align*}
\text{M} + \text{ADP} & \underset{1}{\overset{2a}{\longrightarrow}} \text{M}\text{ADP} & \overset{2b}{\longrightarrow} & \text{M}^1\text{ADP} & \text{M}^2\text{ADP} \\
\text{M}^{\#}\text{ADP} & \overset{\text{Reaction 2}}{\longrightarrow}
\end{align*}
\]

fluorescence quench experienced by Trp\(^{501}\).

The rate constants for ADP dissociation from W129+ and W131+ were determined by ADP displacement by excess mant-ATP as a chaser as 0.8 ± 0.1 s\(^{-1}\) (W129+) and 1.7 ± 0.3 s\(^{-1}\) (W131+). These values are consistent with the upper limits set by the y intercepts of the nucleotide concentration dependence of the slow phase rates extracted from the binding transients.

W129+ fluorescence enhancement on pyrophosphate binding is a much slower process than the quench seen on nucleotide addition. Fig. 7a shows a stopped-flow record of the reaction of W129+ (2 \( \mu M \)) with 100 \( \mu M \) PP\(_i\). The traces were monophasic, and the observed rate constant showed hyperbolic dependence on [PP\(_i\)] (Fig. 7b). \( k_{obs} \) saturated at 0.58 ± 0.02 s\(^{-1}\), reaching half-maximum at 89 ± 11 \( \mu M \).

Upon rapid mixing with ATP, the W129+/W501+ double tryptophan mutant showed an initial large quench followed by a large enhancement (Fig. 8a). The rate of the fast quench phase plotted against [ATP] had an initial slope of 1.80 ± 0.02 \( \times 10^6 M^{-1} s^{-1} \), with an indication of saturation around 500 s\(^{-1}\) (Fig. 8b). The rate of the slow enhancement plateaued at 8 s\(^{-1}\) (Fig. 8c), which indicates that the hydrolysis step in this mutant is slower than in the wild-type (where this value is around 30 s\(^{-1}\)). The traces of the reaction with ADP fitted a monophasic exponential satisfactorily, and the dependence of \( k_{obs} \) on [ADP] had an initial slope of 0.42 ± 0.01 \( \times 10^6 M^{-1} s^{-1} \) with a y intercept of 3.1 ± 0.2 s\(^{-1}\), in agreement with the results of the ADP displacement experiment (not shown).

**DISCUSSION**

Despite the long history (1–3), the assignment of tryptophan fluorescence changes to specific steps in the vertebrate skeletal muscle myosin and actomyosin ATPase pathways has been controversial and difficult to resolve for a number of reasons. At 20 °C, the rapid isomerization steps are lost within the dead time of the stopped flow apparatus, and only the fluorescence change associated with hydrolysis is clearly resolved. At lower temperatures, additional steps were resolved, which suggested that there are at least two binding isomerizations that are...
distinct from the hydrolysis step (3). In the presence of actin,
there are further complications arising from the potential of
tryptophans in actin to contribute to the signal change, pertur-
bations of myosin tryptophans that are sensitive to actin bind-
ing (2, 25), and the technical difficulty of the effect of light
scattering changes on the measured fluorescence signal (26).
Use of *D. discoideum* myosin II constructs has the advantage
that tryptophan residues can be added or removed by mutagen-
esis to probe specific regions of the myosin motor domain. Here we focus on the response of tryptophan residues located near the myosin nucleotide binding site. We show that a tryptophan residue at position 129 or 131 responds with similar kinetics (after allowing for species differences) as previously characterized for rabbit skeletal myosin (3), although the fluorescence change is in the opposite direction. The cause of the fluorescence enhancement on nucleotide binding seen with vertebrate myosins has therefore not been unambiguously identified. There are several candidates for the source of the signal change in vertebrate myosins. First, the two tryptophans in the vicinity of the binding site (Trp113 and Trp131 in rabbit skeletal myosin), which are conserved only among skeletal and cardiac muscle isoforms, are likely to be sensitive to nucleotide binding (6, 8), although their local environments may be different from those in D. discoideum myosin to give a net enhancement rather than quench. Park and Burghardt (21) isolated Trp131 emission in skeletal muscle subfragment 1 by means of elimination of its fluorescence contribution using site-specific chemical modification. Their data show that this side chain indeed responds with a −20% enhancement upon binding of nucleotide, but they excluded Trp131 as the tryptophan sensitive to ATP hydrolysis on the basis that the signal was similar in different nucleotide states. Based on structural data, Trp131 also cannot be excluded as a potential candidate responsible for nucleotide binding-induced signal changes. The contribution of these residues to the signal change is also indicated by the fact that wild-type D. discoideum myosin II, which lacks Trp131 (and Trp113), does not respond with a significant change in fluorescence on nucleotide binding (8, 9). The single tryptophan residues of the D. discoideum myosin mutants presented in this study (Trp113, Trp129, and Trp131) were insensitive to the events of the ATPase cycle other than nucleotide binding. However, the combined contributions of Trp113 and Trp131 would lead to a net quench rather than enhancement on nucleotide binding.

The conservative tryptophan residue in the relay loop of the motor domain (Trp510 in skeletal, Trp512 in smooth muscle, and Trp501 in D. discoideum myosin), which is sensitive to the open-closed transition, also shows a fluorescence change upon nucleotide binding. Skeletal and smooth muscle myosin relay loop tryptophans show 38 and 30% enhancement upon ADP addition, respectively (21, 22). In the W501 mutant, a 15–20% quench can be detected on this step (4, 5). It is unlikely that other tryptophans are involved (e.g., the conserved Trp440 in D. discoideum, Trp440 in skeletal myosin) because the W501F and W501Y mutants show almost no fluorescence change upon adding nucleotide (4, 20). Our data therefore indicate that (i) both the tryptophans(s) near the binding site and the relay loop residue contribute to the fluorescence change upon nucleotide binding prior to the open-closed transition, and (ii) the direction and extent of the fluorescence change of the responsive tryptophans is largely determined by the nonconservative environment surrounding the tryptophan side chain. Thus, we conclude that the global conformational changes undertaken by these myosins are conserved (on the basis of similar rate profiles and functional properties), and even the local movement of particular tryptophan residues could be similar, but the environment experienced by the tryptophan side chains during such movements will vary because of nonconserved residues around them.

The acrylamide quenching experiments indicate that a tryptophan residue at position 129 is solvent-exposed, as expected from the crystal structures (17, 18). Nevertheless, time-resolved anisotropy measurements indicate that this side chain is not freely mobile, and interconversion between rotamer states occurs on a time scale slower than net rotation of the motor domain, as was also noted for TrpP145 (16). Nucleotide binding is accompanied by a protection of Trp129 from collisional quenching by agents in solution, but this residue is now subject to increased static quenching, possibly by direct association with the adenosine moiety.

Regardless of the specific origin of the fluorescence change, the large quench observed on nucleotide binding to W129+ allows a more precise analysis of the mechanism than has hitherto been possible with wild-type proteins. Furthermore, the signal from Trp131 is not sensitive to the open-closed transition, which removes some of the ambiguity in the analysis. This is particularly important because much of the earlier data exploited analogs such as AMP-PNP and ATPγS to mimic the nonhydrolyzed ATP state, but it is now known that these analogs induce the closed state to a limited extent (4, 5, 7) and hence have a contribution from the signal traditionally assigned to the M*ADP·P state (M**ADP·P, in skeletal myosin). ADP has less or no tendency to induce the closed state (5, 7) and is therefore the simplest nucleotide with which to explore the initial binding steps.

In accordance with this concept, the overall profiles of different nucleotides binding to the W129+ construct were similar in character to that observed by Trybus and Taylor (3) for ADP binding to skeletal myosin at 5 °C (at higher temperatures, the isomerization steps with skeletal myosin are too fast to resolve). The profiles of skeletal and D. discoideum myosins differ in detail in the near loss of the slow phase at high [nucleotide], which indicates that the Mγ-L and Mγ·L states have nearly the same fluorescence yield in D. discoideum myosin (hence at saturating [nucleotide], step 2b is optically silent). Furthermore, the kobs of the fast phase appeared to extrapolate back to give a large intercept value (250–1000 s−1), whereas it was close to zero in the Trybus and Taylor (3) data. This aspect is difficult to characterize with accuracy, however, it can be accounted for by an additional weak but competitive nonproductive binding mode of the nucleotide as in Reaction 2. Here, M#·L might represent a nucleotide bound to the active site in a noncompetent orientation that must dissociate and reassociate (or rearrange while bound) before forming the tightly bound form Mγ·L.

The fluorescence transients from the double tryptophan construct, W129+/W501+, show a clear distinction between the nucleotide binding steps and the open-closed transition that is coupled to hydrolysis, because the signals have opposite signs (cf. skeletal myosin, where they both give enhancements). Furthermore, the effective hydrolysis rate is slowed to 8 s−1 at 20 °C, which may be useful for developing conformationally resolved single molecule ATPase assays because the transition occurs on the time scale appropriate for video acquisition (27).

Although the aim of this work was to characterize the tryptophan residue(s) in the motor domain that respond to the nucleotide binding steps and to exploit the fluorescence signal to delineate the elementary steps of this process, it is of interest to relate these steps to those that occur in the presence of actin. Previous studies have estimated that the ATP-induced dissociation of acto-D. discoideum motor domain is limited by a first order step of 150–450 s−1 at 20 °C (8, 9) and is therefore comparable with the slower of the two isomerization steps revealed by Trp129 fluorescence. We confirm that the acto-W129+ construct dissociates with a maximum rate constant of 150 s−1 at saturating [ATP]. The conformational changes sensed by the tryptophan residues near the active site (Trp129 and Trp131) are therefore likely to occur before or are coincident with the ATP-induced actomyosin dissociation reaction. This is in agreement with thermodynamic arguments that require coupling with an
effectively irreversible step (step 2b with ATP) in the binding mechanism to provide sufficient energy to drive the actomyosin dissociation reaction. We find no evidence of a slower tryptophan fluorescence change sensed by Trp129 after actin dissociation, but previous reports with skeletal myosin where this was observed may reflect a contribution from the open-closed transition (26). Single tryptophan-containing constructs will provide a valuable tool for further delineating communication pathways between the actin site, nucleotide site, and converter region of myosins.

Acknowledgments—We thank Dr. Stanley R. Botchway (Rutherford Appleton Laboratory) for assistance in conducting the time-correlated single photon counting measurements.

REFERENCES
1. Bagshaw, C. R., Eccleston, J. F., Eckstein, F., Goody, R. S., Gutfreund, H., and Trentham, D. R. (1974) Biocherm. J. 141, 351–364
2. Johnson, K. A., and Taylor, E. W. (1978) Biochemistry 17, 3432–3442
3. Trybus, K. M., and Taylor, E. W. (1982) Biochemistry 21, 1284–1294
4. Malnási-Csizmadia, A., Woolley, R. J., and Bagshaw, C. R. (2000) Biochemistry 39, 16135–16146
5. Malnási-Csizmadia, A., Pearson, D. S., Kovacs, M., Woolley, R. J., Geeves, M. A., and Bagshaw, C. R. (2001) Biochemistry, 40, 12727–12737
6. Geeves, M. A., and Helmes, K. C. (1999) Annu. Rev. Biochem. 68, 657–728
7. Urbanke, C., and Wray, J. (2001) Biochem. J. 358, 165–173
8. Kurzawa, S., E., Manstein, D. J., and Geeves, M. A. (1997) Biochemistry 36, 317–323
9. Kuhlman, P. A., and Bagshaw, C. R. (1998) J. Muscle Res. Cell Motil. 19, 491–504
10. Kovács, M., Malnási-Csizmadia, A., Woolley, R. J., and Bagshaw, C. R. (2002) Biophys. J. 82, 407 (abstr.)
11. Manstein, D. J., Schuster, H. P., Morandini, P., and Hunt, D. M. (1995) Gene (Amst.) 163, 129–134
12. Trentham, D. R., Barcley, R. G., Eccleston, J. F., and Weeds, A. G. (1972) Biochem. J. 126, 635–644
13. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy, 2nd Ed., Kluwer Academic, New York
14. Peterman, B. F. (1979) Anal. Biochem. 93, 442–444
15. Walsmley, A. R., and Bagshaw, C. R. (1989) Anal. Biochem. 176, 313–318
16. Malnási-Csizmadia, A., Kovacs, M., Woolley, R. J. Botchway, S. W., and Bagshaw, C. R. (2001) J. Biol. Chem. 276, 19483–19490
17. Rayment, I., Rypniewski, W. R., Schmidt-Base, K., Smith, R., Tomchick, D. R., Benning, M. M., Winkelmann, D. A., Wesenberg, G., and Holden, H. M. (1993) Science 261, 50–58
18. Galli A. M., Rauer C. B., Thoden J. B., and Rayment, I. (1997) Biochemistry 36, 11619–11628
19. Sellers, J. R. (1999) Myosins (2nd Ed.) Oxford University Press, Oxford
20. Batra, R., and Manstein, D. J. (1999) Biol. Chem. 380, 1017–1023
21. Park, S., and Burghardt, T. P. (2000) Biochemistry 39, 11732–11741
22. Yengo, C. M., Chrin, L. R., Rovner, A. S., and Berger, C. L. (2000) J. Biol. Chem. 275, 25461–25467
23. Onishi H, Konishi, K., Fujiwara, K., Hayakawa, K., Tanokura, M., Martinez, H. M., and Morales, M. F. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11203–11208
24. Smith, C. A., and Rayment, I. (1995) Biochemistry 34, 8973–8981
25. Yengo, C. M., Chrin, L., Rovner, A. S., and Berger, C. L. (1999) Biochemistry 38, 14515–14523
26. Millar, N. C., and Geeves, M. A. (1988) Biochem. J. 249, 735–743
27. Bagshaw C. R., and Combeur, P. B. (2000) Single Mol. 1, 269–275