Structural kinetics of myosin by transient time-resolved FRET

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For many proteins, especially for molecular motors and other enzymes, the functional mechanisms remain unsolved due to a gap between static structural data and kinetics. We have filled this gap by detecting structure and kinetics simultaneously. This structural kinetics experiment is made possible by a new technique, (TR)2FRET (transient time-resolved FRET), which resolves protein structural states on the millisecond timescale during the transient phase of a biochemical reaction. (TR)2FRET is accomplished with a fluorescence instrument that uses a pulsed laser and direct waveform recording to acquire an accurate subnanosecond time-resolved fluorescence decay every 0.1 ms after stopped flow. To apply this method to myosin, we labeled the force-generating region site specifically with two probes, mixed rapidly with ATP to initiate the recovery stroke, and measured the interprobe distance by (TR)2FRET with high resolution in both space and time. We found that the relay helix bends during the recovery stroke, most of which occurs before ATP is hydrolyzed, and two structural states (relay helix straight and bent) are resolved in each nucleotide-bound biochemical state. Thus the structural transition of the force-generating region of myosin is only loosely coupled to the ATPase reaction, with conformational selection driving the motor mechanism.

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Structural kinetics lies at the heart of protein function. Transitions among distinct structural states, each characterized by both structural order and internal dynamic disorder, are typically required for the function of a protein, especially an enzyme. To describe the mechanism of a protein’s function is to describe its structural kinetics, i.e., the coupling of protein structural transitions to biochemical kinetics, as defined by changes in bound ligand (1–3). However, for most proteins there remains mechanistic ambiguity due to a gap between structural data, determined primarily from static protein crystals, and kinetics, measured during the transient phase of the biochemical reaction. In the present study, we have closed this gap by measuring structure and kinetics simultaneously, using myosin as a powerful example.

In a molecular motor, ATP binding and hydrolysis initiate protein structural changes that lead to force generation, and characterization of motor protein structural kinetics is essential to understand the protein in action. Myosin is a molecular motor responsible for actin-dependent force generation and movement in muscle and nonmuscle cells; it works cyclically, producing mechanical work on actin using energy from ATP hydrolysis (recently reviewed in refs. 4 and 5). Transient kinetics in myosin has been typically monitored by tryptophan fluorescence (6, 7), but that signal provides no direct structural information. FRET does provide structural information, in the form of interprobe distance, and transient FRET experiments have provided a glimpse of structural kinetics in muscle and other proteins (8–10). However, previous transient FRET measurements have been limited to the monitoring of a single signal with continuous excitation and detection, whereas only nanosecond time-resolved FRET (TR-FRET), in response to pulsed excitation, can resolve multiple distances and quantitate disorder (11, 12). Therefore, we have developed a spectroscopic technique, transient time-resolved FRET (TR2FRET), in which a complete and accurate time-resolved fluorescence decay is recorded every 0.1 ms after stopped flow, 102 times faster than previously achieved (13). This method unambiguously resolves the kinetics of protein structural states (conformations) on the millisecond timescale. This approach is versatile and can be applied to a wide range of biological systems, revealing another dimension of insight into structural dynamics. In the present study, we have used it to resolve structural transitions within the force-generating region of myosin following the addition of ATP.

In the current working model for myosin’s mechanism, the myosin head undergoes major structural changes twice during the ATPase cycle, producing the power stroke and the recovery stroke. Although numerous structures of myosin have been captured by X-ray crystallography (4), to a good approximation there are two principal classes of nucleotide-bound conformations that show significant differences in the overall shape of the head and in the force-generating region (14). These two structural states are designated M* (green) and M** (red) in Fig. 1. The recovery stroke is the transition from M* to M**, in which the entire head bends (Fig. 1A) and the relay helix undergoes a remarkably similar bend (Fig. 1B). Based on nucleotide analogs used to produce the crystal structures, and on Trp fluorescence changes observed in solution, a minimal kinetic mechanism is shown in Scheme 1, which suggests that there is a tight coupling between the biochemical state (defined by the bound nucleotide) and the structural state of myosin.

However, there is growing evidence that the coupling between biochemical and structural states in myosin is not tight (reviewed in ref. 4). Two different crystal structures of the myosin head can be trapped with a single nucleotide analog (14–16). Change in the intrinsic fluorescence of a myosin-nucleotide analog complex upon pressure and temperature jumps suggests a transition between two structural states of myosin (17). Spectroscopic data on myosin with probes at the active site (18, 19), in the force-generating region (11, 20, 21), or in the actin-binding cleft (22) show evidence for two resolved structural states (conformations) in the presence of a single biochemical state (bound ligand). TR-FRET has provided a clear structural picture of this phenomenon: With a single nucleotide analog bound to myosin, two distinct structural states (M* straight and M** bent) were resolved for the entire head (23) (Fig. 1A) or for the relay helix in the force-generating region (11) (Fig. 1B). All these data suggest...
the simultaneous presence of two distinct structural states of myosin, $M^*$ and $M^{**}$, in one biochemical state, with the ATP hydrolysis step changing only the relative populations (mole fractions $X^*$ and $X^{**}$) of $M^*$ and $M^{**}$. However, the assignment of these trapped nucleotide analog states to specific biochemical intermediates in the kinetic cycle is uncertain. Analysis of myosin’s intrinsic fluorescence kinetics does suggest a branched mechanism of myosin–nucleotide interaction with two structurally different intermediates (5, 6, 17, 24), but this method does not have the structural resolution of TR-FRET. In order to obtain simultaneous structural and kinetic resolution, the present study uses (TR)$^2$FRET (performing a complete TR-FRET experiment every 0.1 ms) to determine directly the structural kinetics within the myosin force-generating region during the myosin–ATP interaction and the recovery stroke (steps 1 and 2, Scheme 1 and Fig. 1A).

**Results**

**Relay Helix Structural Transitions Induced by the Myosin-ATP Interaction, Resolved in Real Time.** We engineered two double-cysteine (Cys) mutants in a Cys-lite myosin construct, which had all reactive native cysteines removed (23). In each mutant, one labeling site is located at the C terminus of the relay helix (K498C) and another within the stable helices on the lower 50 kDa domain (D515C or A639C) (Fig. 1B). We labeled these cysteine residues with a donor–acceptor optical pair [(5-((2-((iodoacetyl)amino)-ethyl)amino)naphthalene-1-sulfonic acid) (IAEDANS), (4-((4-(dimethylamino)phenyl)azo)benzoic acid) (DABCYL)] (11). At equilibrium, with nucleotide analogs bound, the interprobe distance distribution was determined with both pulsed electron paramagnetic resonance and time-resolved FRET, with the resulting distances in good agreement with those measured from crystal structures, but both $M^*$ (relay helix straight) and $M^{**}$ (bent) structures were observed simultaneously (11).

To resolve transitions between myosin structural states with millisecond kinetic resolution during the recovery stroke, we designed and constructed the (TR)$^2$FRET fluorometer (Fig. 1C). This instrument is based on our recently reported high-performance time-resolved fluorescence (HPTRF) instrument, which improves the throughput for time-resolved fluorescence by a factor of $10^5$ (13). Solutions of 5-mM ATP and 10–30-μM donor–acceptor labeled myosin (syringe concentrations) were mixed in the fluorometer, and the complete time-resolved donor fluorescence decay was acquired after each laser pulse (10,000 times per second) immediately after myosin–ATP mixing (dead time 1.4 ms). The result is a two-dimensional dataset, resolved on both the nanosecond fluorescence decay timescale (0.125 ns per point) and the millisecond biochemical reaction timescale (0.1 ms per point) (Fig. 1D). At each time point in the millisecond-resolved transient, (TR)$^2$FRET resolved distinct structural states of myosin, with essentially the same high resolution and precision as in previous equilibrium TR-FRET experiments (11). In TR-FRET, the decay rate of donor fluorescence increases with the inverse sixth power of the donor–acceptor distance $R$ (Eq. 2). We fitted donor fluorescence decays globally as the sum of exponentials.

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(Eqs. 1–4), assuming two structural states, pre- and postrecovery (M* and M**), corresponding to two interprobe distances, R1 and R2, each with a distinct Gaussian distance distribution (Eq. 3 and Fig. 1E). The best fit to the data required two structural states; i.e., two states gave a better fit than one, but three states did not improve the fit. An excellent fit was obtained by assuming that these myosin structural states do not change during the course of the myosin–ATP interaction, but their populations (X+ and X−) do. After myosin–ATP mixing, the mole fraction X− of myosin in the M** structural state increases, as X+ decreases (Fig. 1F).

By inspection of Fig. 1F, this recovery stroke is clearly biphasic, with one time constant of several milliseconds and the other at least 10 times slower. Kinetic traces for X+ and X− populations were fitted by a system of differential equations (Eq. 5), corresponding to Scheme 2, assuming that the transition from the postrecovery stroke M**.D.P state (k4) is the rate-limiting step of the reaction (25).

The resulting rate constants are shown in Table 1. The first step in Scheme 2, ATP binding, is very fast, practically irreversible, and temperature independent (k1 and k−1, Table 1). The recovery stroke (M*.T → M**.T), characterized by k2 and k−2, and the hydrolysis step (M**.T → M**.D.P), characterized by k3 and k−3, are temperature dependent. The recovery stroke, marked by the bending of the relay helix, is more than an order of magnitude faster than ATP hydrolysis. The relay helix undergoes this structural transition within a few milliseconds after ATP binding, with a rate in agreement with that of fluorescence changes of single tryptophan mutant at active site, previously attributed to ATP binding (26). These results show that ATP binding plays the crucial role in the recovery stroke, contradicting the classic model of myosin function, in which ATP hydrolysis is tightly coupled to the recovery stroke and serves as its driving force (reviewed in ref. 4).

ATP hydrolysis is a relatively slow process, increasing the bent population (M**) of the relay helix only indirectly by mass action, converting M**.T to M**.D.P (step 3 in Scheme 2). The recovery stroke precedes ATP hydrolysis, which is needed not to fuel the recovery stroke but to facilitate product release and continue the cycle.

Energy Landscape of Myosin Structural Transitions. The temperature dependence of rate constants was interpreted according to transition state theory (27) assuming linear temperature dependence for thermodynamic parameters (an approximation for myosin in this temperature range). The free energy of activation for each step in Scheme 2, as well as the corresponding enthalpy and entropy, were fitted by a system of differential equations (Eq. 5), corre-
must lower the energy barrier, bringing directionality to the scheme. The present study is focused on structural changes in the force-generating region of myosin during the recovery stroke. A complete description of the myosin mechanism will require future studies on other phases of the myosin ATPase cycle (especially the power stroke, the force-generating reversal of the recovery stroke, induced by actin) and on other regions of the myosin motor, such as nucleotide and actin-binding sites of myosin.

In conclusion, in this work we introduce transient time-resolved FRET—a powerful tool that makes possible the direct analysis of structural kinetics. The key feature of (TR)²FRET is the resolution of protein structural states (Fig. 1E) with submillisecond time resolution (Fig. 1E). This technical advantage allowed us to monitor distinct structural states of the relay helix in the myosin molecular motor directly during the recovery stroke. The significance of this space-time resolution is threefold. First, our experiments directly resolved two structural states of myosin that were occupied in a single biochemical state, as determined by the bound nucleotide. Second, we were able to define not only the mean interprobe distance in each state but also the substantial disorder (presumably related to configurational entropy) present in each structural state. Finally, the mole fraction of each protein structural state was measured directly with millisecond resolution, leading to unambiguous determination of the kinetics and thermodynamics of the system, revealing how structural and biochemical changes are coupled. This study demonstrates the power of the (TR)²FRET technique, revealing real-time protein structural kinetics induced by protein–ligand interactions.

**Materials and Methods**

**Protein Preparation and Labeling.** Mutants of Cys-lite Dictyostelium discoideum myosin motor domain were constructed, expressed, and purified as described previously (33). For FRET measurements, the protein (50 μM) was first incubated with donor (45 μM IAEDANS, Invitrogen) for 12 h on ice, then protein was diluted to 25 μM and incubated for 2 h on ice with 100-μM acceptor (DABCYL-C2-maleimide, Anaspec). After each labeling step, unreacted label was removed with size-exclusion spin columns (Pierce). Labeling buffer contained 20 mM MOPS (pH 7.5), 50 mM KCl, 6 mM MgCl₂, and 1 mM EDTA. Complexes of myosin with nucleotide analogs were prepared as described previously (20).

**ATPase Assays.** Myosin ATPase activity was measured (T = 25 °C in 10 mM Tris, 6 mM MgCl₂, 5 mM ATP, pH 7.5) in the presence and absence of actin, by the liberation of inorganic phosphate (34). The dependence of myosin ATPase activity on actin concentration was fitted to determine Vₘₐₓ (activity at saturating actin) as reported in Table 2. As reported previously (11), both basal and actin-activated ATPase activities were comparable (within a factor of 2) between unlabeled and labeled proteins and were also comparable (within a factor of 2) to values reported for other Dictyostelium myosin constructs (20, 22, 33), indicating that neither mutations nor labeling caused significant effects on myosin catalytic activity (Table 2).

**TR²FRET Experiments.** TR-FRET experiments were performed with an HPTFR instrument, utilizing direct wavelet recording as described previously (11, 13). Fluorescence of IAEDANS-myosin was excited with the third harmonic (355 nm) of a passively Q-switched microchip yttrium aluminum garnet laser (NanoUV-355, JDS Uniphase), operated at a pulse repetition frequency of 10 kHz, and selected with a 420-nm long-pass glass filter. To avoid anisotropy effects, fluorescence was passed through a polarizer oriented at the magic angle. Fluorescence signals were detected after every laser pulse with a multimultiplier module (Hamamatsu H7773-20, rise time 0.78 ns) and acquired with a transient digitizer (Acqiris DC252) with time resolution of 0.125 ns (8 G5/s). The instrument response function (IRF) was acquired with scattered light at the same instrumental settings as in the fluorescence measurement, except that there was no emission filter and the emission polarization was vertical. To obtain (TR)²FRET data, a stopped-flow apparatus (SFM-20, Bio-Logic) was attached to the fluorometer described above to measure transient kinetics of the myosin–ATP reaction. Myosin and ATP solutions were loaded into syringes, and the total flow rate through the mixer was 8 mL/s, giving a dead time of 1.4 ms. Donor fluorescence waveforms, consisting of 500 points (each having S/N ≥ 100) spaced 0.125 ns apart, were acquired after every laser pulse (10 kHz). All fluorescence experiments were performed in a buffer containing 20 mM 4-(2-hydroxyethyl)-1-piperazinopropanesulfonic acid, 6 mM MgCl₂, 1 mM EGTA, pH 8.0.

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<tr>
<th>Biochemical state (bound ligand)</th>
<th>apo</th>
<th>T</th>
<th>D.P</th>
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<tr>
<td>Structural state</td>
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**Scheme 3.** Full scheme of myosin ATPase cycle. M, myosin; T, ATP; D, ADP; and P, inorganic phosphate. Structural states are populated differently in different biochemical states. States with clearly detected populations are shown in dark green. Light green shows required intermediate states. Green arrows show the most probable course of the reaction, with steps numbered as in Scheme 2. The biochemical state of myosin, determined by bound nucleotide, is loosely coupled to the structural state (M, M*, and M**).
Analysis of TR2FRET Data. The observed donor-only waveform $F_{DA}(t)$ was fit by a simulation $F_{DA}(t)$, consisting of a multiplexponential decay $F_{D}(t)$ convolved with the IRF (from light scatter):

$$F_D(t) = \sum_{i=1}^{\infty} A_i \exp(-t/\tau_D),$$

$$F_{DA}(t) = \int_{-\infty}^{\infty} F_D(t') \mathrm{IRF}(t-t') dt',$$  \[1\]

where $\tau_D$ are donor-only fluorescence lifetimes. We found that $n = 2$ in Eq. 1 was sufficient, i.e., $n = 3$ did not reduce the residual or $\chi^2$. The fluorescence signal of donor-acceptor labeled myosin, $F_{DA}(t)$, was fitted by assuming that the only change in the donor fluorescence was increased rate due to energy transfer:

$$F_{DA}(t) = \int_{-\infty}^{\infty} \rho(R) \cdot \sum_{i=1}^{\infty} A_i \exp((-t/\tau_{DA})(1 + [R_{fit}/R_0^i])} dR.$$

where $A_i$ and $\tau_{DA}$ were determined from the fit to Eq. 1, and the Förster distance $R_0$ was determined as previously (11). The probability distribution $\rho(R)$, a sum of Gaussian functions, takes on values for flexibility:

$$\rho(R) = \sum_{i=1}^{\infty} X_i \sigma_i^{-1}(2\pi)^{1/2} \exp((-[(R-R_i)^2/2\sigma_i)]),$$

$$\sigma_i = \text{FWHM}/3 \times (2 \ln 2)^{1/2}.$$  \[3\]

After addition of ATP, the best fit was consistently obtained for $m = 2$, corresponding to two structural states (11): $M'$ (pre-recovery, straight relay helix), having molecule fraction $X'$, distance $R'$, and width FWHM$^*$; and $M''$ (post-recovery, bent relay helix), having molecule fraction $X''$, distance $R''$, and width FWHM$^{**}$. The observed waveform $F_{DA}(t)$ was fit by $F_{DA}(t)$:

$$F_{DA}(t) = X_D F_D(t) + (1 - X_D) F_{DA}(t),$$

$$F_{DA}(t) = \int_{-\infty}^{\infty} \mathrm{IRF}(t-t') \cdot F_{DA}(t') dt',$$  \[4\]

where $X_D$ is the fraction of donor-labeled proteins lacking acceptor. Each set of fluorescence waveforms from a (TR2)FRET experiment was fit globally to Eq. 4. Amplitudes $A_i$, lifetimes $\tau_D$, distances $R'$ and $R''$, widths FWHM$^*$ and FWHM$^{**}$, and the fraction of donor-labeled myosin $X_0$ were linked and varied simultaneously for all waveforms during the global fit. Only $X''$ (the mole fraction of the $M''$ structural state) was allowed to vary independently for every waveform (every kinetic time point). The fraction $X_0$ of donor-only labeled myosin mutant was allowed to vary between different protein preparations.

Analysis of Recovery Stroke Kinetics. To determine kinetic constants of the myosin-ATP reaction, the time-dependent trace of the $M''$ mole fraction $X'',$, obtained by fitting the (TR2)FRET data as described in Eqs. 2-4 was fitted according to the solution of the system of differential equations for $M''$ (Eq. 5):

$$dM'[t]/dt = -k_1[M][T] + k_1[M'.T]$$

$$dM'[T]/dt = k_1[M'.T] - k_2[M'.T] - k_3[M''.T] + k_2[M'''.T].$$

$$dM''[T]/dt = k_2[M'.T] - k_2[M''.T] - k_3[M'''.T]$$

$$+ k_3[M'''.D.P]$$

$$dM''[D.P]/dt = k_3[M'''.T] - k_3[M'''.D.P].$$  \[5\]

In Eq. 5, molecular species and rate constants are defined as in Scheme 2. Each equation describes the time evolution of each myosin state. Structural states are indicated by $M, M', M''$; biochemical states are defined by the bound ligand (apo, T, D.P). Positive and negative terms describe the increase and decrease of structural state concentration due to forward and reverse transitions in Scheme 2. The time dependence of system was solved in Wolfram Mathematica 5.1 using the NDSolve routine. The fit to experimental data was realized with the NMinimize routine, using the Differential Evolution algorithm, with 10,000 initial parent points, scaling factor 1, and cross probability 0.8.

The temperature dependence of rate constants was analyzed in terms of transition state theory (27). The free energy of activation was determined from

$$\Delta G_a = -RT \ln(k_h/k_iT) = \Delta H_a - T \Delta S_a.$$  \[6\]

where $h$ is Planck’s constant, $k_i$ is Boltzmann’s constant, R is the gas constant, and $T$ is the temperature. The enthalpy and entropy of activation, $\Delta H_a$ and $\Delta S_a$ were obtained from the temperature dependence of $\Delta G$ (Eq. 6).

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