Kinetic Characterization of Tail Swing Steps in the ATPase Cycle of Dictyostelium Cytoplasmic Dynein*

Received for publication, March 5, 2007, and in revised form, May 31, 2007 Published, JBC Papers in Press, June 4, 2007, DOI 10.1074/jbc.M701914200

Toshifumi Mogami1, Takahide Kon1, Kohji Ito2, and Kazuo Sutoh1†

From the 1Department of Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Komaba 3-8-1, Meguro-ku, Tokyo 153-8902, Japan and 2Department of Biology, Chiba University, Inage-ku, Chiba 263-8522, Japan

According to the power stroke model of dynein deduced from electron microscopic and fluorescence resonance energy transfer studies, the power stroke and the recovery stroke are expected to take place at the two isomerization steps of the ATPase cycle at the primary ATPase site. Here, we have conducted presteady-state kinetic analyses of these two isomerization steps with the single-headed motor domain of Dictyostelium cytoplasmic dynein by employing fluorescence resonance energy transfer to probe ATPase steps at the primary site and tail positions. Our results show that the recovery stroke at the first isomerization step proceeds quickly (∼180 s⁻¹), whereas the power stroke at the second isomerization step is very slow (∼0.2 s⁻¹) in the absence of microtubules, and that the presence of microtubules accelerates the second but not the first step. Moreover, a comparison of the microtubule-induced acceleration of the power stroke step and that of steady-state ATP hydrolysis implies the intriguing possibility that microtubules simultaneously accelerate the ATPase activity not only at the primary site but also at other site(s) in the motor domain.

Dynein is a multisubunit microtubule-based motor protein that utilizes ATP hydrolysis to power the beating of cilia and flagella as well as minus-end directed transport of cargoes on microtubules (MTs) in eukaryotic cells (1–6). The dynein heavy chain, a primal component of the dynein complex, is composed of three distinct structural units, the tail, head, and stalk (7, 8). The N-terminal one-third of the heavy chain forms the slender tail, which contains a dimerization site and binding sites for associating polypeptides such as intermediate, light-intermediate, and light chains (9–13). The head is composed of six AAA+ modules arranged into a ring-like structure common to AAA+ family proteins (14–16). Four of these AAA+ modules (AAA1–AAA4) contain ATP binding/hydrolysis sites (17–19). Among these multiple ATP binding/hydrolysis sites, AAA1 site is likely the primary ATPase site directly responsible for the generation of force by dynein (20–22). The stalk residing between the AAA4 and AAA5 is composed of a long coiled-coil and a small globular tip, which is thought to be the binding site for MTs (8, 23, 24).

The molecular model of force generation by dynein has begun to emerge only recently (16, 22, 25, 26). Electron microscopic image analyses of an axonemal dynein have suggested that the dynein tail adopts two distinct positions against the head during the ATPase cycle (16). Based on these electron microscopic images, it has been proposed that the tail swings between these two positions in an ATPase-dependent manner to generate the power stroke that permits the active movement of dynein on MTs (16). Steady-state fluorescence resonance energy transfer (FRET) measurements of the dynein motor domain with GFP-based FRET sensors have also shown that the tail adopts two distinct positions, presumably the post-stroke and the pre-stroke positions, depending on the nucleotide states of the AAA1 ATPase site (22). Furthermore, the coupling of these two tail positions and intermediate states of the ATPase cycle has been established by FRET measurements on motor domains that are trapped in specific intermediate states by means of nucleotides and/or mutations (22). These studies have led to a tail swing model that describes how the tail position changes depending on ATPase steps (22) (Fig. 1).

In the tail swing model, the ATPase cycle is assumed to start with the rapid binding of ATP to dynein. Upon the ATP binding, dynein adopts the D-ATP state and then the D*-ATP state after isomerization (Fig. 1A). Here, dynein in which the tail is in the post-stroke position is designated as D, whereas dynein in which the tail is in the pre-stroke position is termed D* (Fig. 1B). At this first isomerization step the tail swings from the post-stroke position to the pre-stroke position. ATP hydrolysis follows this isomerization step, and dynein adopts the D*-ADP-Pi state. After Pi release, dynein then enters the D*-ADP state (27). Dynein in the D*-ADP state very slowly undergoes a second isomerization to enter the D-ADP state (27, 28). At this step the tail swings from the pre-stroke to the post-stroke position (22). After ADP release, dynein is ready to repeat the full ATPase cycle. In this cycle, the power stroke is presumed to occur at the second isomerization step and the recovery stroke at the first isomerization step.

This tail swing model was deduced mainly from steady-state FRET measurements on “trapped” intermediate states of the ATPase cycle. Therefore, the model should be further tested in...
Kinetics of Dynein Swing Steps

presteady-state kinetic studies that can directly assess the transitions between intermediate states. Here, by employing the FRET signal as a probe for tail positions as well as for ATPase steps at the primary ATPase site, we have monitored the power stroke and recovery stroke steps of the single-headed motor domain of *Dictyostelium* cytoplasmic dynein. Our results address how these two critical steps of the force generation cycle are coupled with the ATPase steps.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Preparation**—Construction of the expression plasmid encoding the 380-kDa cytoplasmic dynein motor domain of *Dictyostelium discoideum* fused with GFP and BFP (HG380B2) was described previously (22). Briefly, His$_6$-GFP (red-shifted GFP) was fused at the N terminus of the motor domain (Val-1383—Ile-4725) through Gly-Gly-Gly-Lys as a linker. The BFP coding region (Val-2—Lys-238) of EBFP (Clontech) was inserted between codons encoding Ser-2471 and Ser-2472, residues expected to lie between the AAA2 and AAA3 modules. After confirmation by DNA sequencing, the construct was subcloned into the expression vector MB38 for tetracycline-regulated expression in *Dictyostelium* cells derived from the Ax2 strain, in which expression of the encoded protein is strongly suppressed if tetracycline is added to the culture (29). The plasmid was introduced into *Dictyostelium* cells by electroporation, and transformed cells were selected in HL5 medium supplemented with 10 μg/ml blasticidin S, 10 μg/ml G418, and 10 μg/ml tetracycline. Expressed protein was purified as previously described (30). Tubulin was purified from porcine brain as previously described (31) and kept frozen in liquid N$_2$ until use. For preparation of paclitaxel-stabilized MTs, tubulin was polymerized by incubation at 37°C for 30 min in the presence of 0.5 mM GTP and 1 mM MgSO$_4$ followed by the addition of 12 μM paclitaxel, and the samples were kept at room temperature. Before use for experiments, the MTs were pelleted by ultracentrifugation at 239,000 × g through 0.5 ml of 25% sucrose cushion and resuspended in assay buffer (10 mM PIPES-KOH, pH 7.0, 50 mM potassium acetate, 4 mM MgSO$_4$, 1 mM EGTA, and 1 mM dithiothreitol) supplemented with 20 μM paclitaxel. Protein concentrations were determined by the Bradford method (Coomassie protein assay reagent; Pierce) using bovine serum albumin as the standard.

**Presteady-state Fluorescence Measurements**—Presteady-state changes in the FRET signal of HG380B2 were followed with an SX-18MV stopped-flow spectrophotometer (Applied Photophysics). Light was provided by a 150-watt xenon arc lamp and passed through a SpectraKinetic monochromator. Time courses of changes in the FRET signal were followed by measurement of GFP fluorescence exciting BFP at 380 nm and monitored at 90° through a 515-nm long-pass filter. Assays were performed in the assay buffer at 25°C. The final concentration of HG380B2 after mixing was 60 nM unless otherwise mentioned. Dynein solution was supplemented with 0.1 mM ADP in the syringe before mixing unless otherwise noted. For measurements in the presence of MTs, the paclitaxel-stabilized MTs were loaded into both sample syringes of the stopped-flow apparatus at the same concentration unless otherwise noted.

The post-stroke to pre-stroke transition was monitored after mixing various concentrations of ATP (0–1 mM, after mixing) into the dynein solution. The pre-stroke to post-stroke transition was monitored after the addition of 50 μM glucose and an excess (~750 units/ml, after mixing) of hexokinase to dynein solutions preincubated with excess ATP.

Raw records taken from stopped-flow experiments in the presence of MTs contained significant noise that was independent of the presence of dynein or ATP and was expected to be a flow birefringence artifact. Thus, data obtained by mixing only MTs was subtracted from the raw data of fluorescence changes in dynein before analyses.

It should be emphasized that the present dynein construct with the GFP-BFP FRET sensors actually reported FRET signals given the fact that the spatial locations of GFP and BFP in the motor domain did not allow intramolecular contact, in contrast to a myosin motor domain fused with similar GFP-BFP FRET sensors. This myosin construct was reported to allow detection of the intramolecular association—dissociation of GFP and BFP, but not FRET signals, because the two fluorescent proteins were close enough to allow direct contact (32).

**Measurement of MT-activated Overall Steady-state ATPase Activity**—The basal and MT-activated ATPase activities of HG380B2 were measured in the assay buffer containing 250 μM ADP in the stopped-flow apparatus using the EnzChek phosphate assay kit (Molecular Probes). ADP was added here because it was rapidly produced in the FRET measurements of the pre-stroke to post-stroke transition by hexokinase. Although there was a report saying that ADP is an inhibitor of dynein ATPase (27), virtually no inhibition was observed on the ATPase activity of HG380B2 in the absence of MTs and in the presence of 10 μM MTs when ADP up to 500 μM was added before the addition of ATP. HG380B2 (20–40 nM), various concentrations of paclitaxel-stabilized MTs (0–20 μM), 2 units/ml purine nucleoside phosphorylase, 0.4 mM 2-amino-6-mercaptop-7-methylpurine riboside, and 500 μM ADP were incubated in one syringe, and then they were rapidly mixed with same volume of 2 mM ATP. Absorbance at 360 nm was continuously monitored and plotted against time. Steady-state phosphate release rate was calculated from the slope of the plot. The concentration of MTs is expressed as that of tubulin dimer throughout this paper.

**RESULTS**

**Kinetics of the Post-stroke to Pre-stroke Transition Probed by FRET**—In the tail swing model shown in Fig. 1, the post-stroke to pre-stroke transition is expected to occur at the first isomerization step D-ATP → D*-ATP. To determine the rate of this isomerization step, we followed the FRET signal after rapidly mixing the motor domain fused with GFP-BFP FRET sensors (HG380B2) and ATP (Fig. 2A). The apparent rate of the transition was obtained by fitting the time course to a single exponential. The rates of the pseudo first-order reaction, thus, obtained at varying ATP concentrations showed a hyperbolic ATP concentration dependence (Fig. 2B).

3 S. A. Burgess, personal communication.
This ATP concentration dependence of the apparent rate was analyzed by a kinetic scheme of the tail swing shown in Fig. 1B. Here, we assumed three intermediate states in Fig. 1A, D*-ATP, D*-ADP-Pi, and D*-ADP, to be a single kinetic state since they are indistinguishable in terms of the FRET signal, and moreover, they are in rapid equilibrium due to slow rates of the steps D*-ATP → D-ATP and D*-ADP → D-ADP as shown later. This kinetic state is designated as D*-N, where N represents any of three bound nucleotides (ATP, ADP-Pi, and ADP).

The first step in the scheme is the rapid formation of the initial collision complex of dynein and ATP, defined by the equilibrium constant $K_1$. The second step is the first isomerization whereby the tail undergoes the post-stroke to pre-stroke transition. The third step is the second isomerization, i.e. the pre-stroke to post-stroke transition, which is a very slow and virtually irreversible step in the absence of MTs. It should be noted that this kinetic scheme represents events at the primary ATPase site (AAA1 ATPase site) responsible for the FRET change (22).

From this scheme the apparent rate of the post-stroke to pre-stroke transition, $k_{obs}(post-pre)$, is described by the equation

$$k_{obs}(post-pre) = K_1 k_2 [ATP] / (1 + K_1 [ATP]) + k_{-2} \quad (Eq. 1)$$

under the conditions that the second isomerization is sufficiently slow. In Fig. 2B, at low concentrations of ATP the observed pseudo-first-order rate was linearly dependent on ATP concentration and yielded an apparent second-order rate constant $K_1 k_2$ of $2.2 \times 10^6 \text{M}^{-1} \text{s}^{-1}$. Above 500 μM ATP, the rate approached the plateau of the maximum rate ($k_2 + k_{-2} = 184 \pm 2 \text{ s}^{-1}$).

We then measured the plateau amplitude of the change in the FRET signal upon the addition of ATP (Fig. 3A). This amplitude corresponds to the proportion of HG380B2 molecules in the pre-stroke state after the isomerization step. The amplitude was normalized by the value obtained when a saturating amount of ATP was added, and the normalized ΔF values (%) were plotted against ATP concentration (Fig. 3B). Under the conditions that the second isomerization step is very slow, ΔF values have a hyperbolic ATP concentration dependence that is represented as

$$\Delta F = 100 K_1 k_2 [ATP] / (1 + K_1 [ATP]) \quad (Eq. 2)$$

where $K_2 = k_2 / k_{-2}$. From the plot, $K_1 k_2$ was estimated as $5.5 \times 10^5 \text{ M}^{-1}$. Because $K_1 k_2$ was $2.2 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ as shown above, $k_{-2}$ was calculated as $4.0 \text{ s}^{-1}$. From this rate of the reverse step of the post-stroke to pre-stroke transition, the rate of the forward step ($k_2$) was calculated as $180 \text{ s}^{-1}$.

Kinetics of the Pre-stroke to Post-stroke Transition—Next, we tried to measure the rate $k_3$ of the second isomerization (Fig.
Kinetics of Dynein Swing Steps

FIGURE 4. Kinetics of the pre-stroke to post-stroke transition after sudden depletion of ATP by hexokinase. A, time courses of changes in GFP fluorescence upon the addition of various concentrations of hexokinase to 60 nM HG380B2 to deplete free ATP. The data are fitted to single exponentials, giving apparent rates. Concentrations of hexokinase are after mixing. B, the apparent rates plotted against hexokinase concentration, giving $k_{obs}(pre-post)$. Error bars are $\pm 1$ S.D.

MTs Accelerate the Pre-stroke to Post-stroke Transition but Not the Post-stroke to Pre-stroke Transition—To determine whether MTs accelerate the first isomerization step, HG380B2 was preincubated with MTs, and 1 mM ATP was rapidly added to the mixture (the concentration of ATP is after mixing). The time course of the change in the FRET signal due to the pre-stroke to post-stroke transition well fitted a single exponential as shown in Fig. 5A. At the high concentration of ATP used here, the observed rate ($k_{obs}(post-pre)$) was almost equal to $k_2$. As shown in Fig. 5B, this rate was virtually independent of MT concentration, especially at higher levels. This result shows that MTs do not significantly affect the post-stroke to pre-stroke transition of the first isomerization step.

In contrast to the first isomerization step, the rate of the second isomerization step in Fig. 1B is expected to increase in the presence of MTs because this step is rate-limiting in the ATPase cycle (22, 27), which will be activated in the presence of MTs (28). We, therefore, examined if MTs accelerate the pre-
stroke to post-stroke transition by using hexokinase to deplete free ATP as above. HG380B2 and MTs were preincubated with a saturating amount of ATP (500 μM), and an excess amount of hexokinase was then rapidly added to the mixture to suddenly deplete free ATP. The time course of the decrease in the FRET signal due to the spontaneous pre-stroke to post-stroke transition (lower curve in Fig. 5C) was fitted to double exponentials, providing two different rates. The slow drift of the baseline gave rise to a minor slow component. The major component corresponded to the pre-stroke to post-stroke transition. Its rate was linearly dependent on MT concentration and reached 15 s⁻¹ with 10 μM MTs (Fig. 5D), showing that the pre-stroke to post-stroke transition is indeed accelerated by MTs.

The pre-stroke to post-stroke transition in the presence of MTs is composed of the reverse step of the first isomerization and the forward step of the second isomerization, as in the case of reactions without MTs. However, the former reaction should be independent of MTs, since dynein is most likely to be released first from MTs and then to isomerize for a successful recovery stroke. Therefore, the MT-dependent increment in the rate of the pre-stroke to post-stroke transition upon the sudden depletion of free ATP would result from an acceleration of the second isomerization alone. It should also be noted that the observed overall rate of the pre-stroke to post-stroke transition contained a large contribution from the MT-independent reverse step of the first isomerization (k₁₋₂ = 4.0 s⁻¹). Thus, instead of the overall rate of the pre-stroke to post-stroke transition, we use this MT-dependent increment represented as the apparent second-order rate constant Kk₃MT, where K is the apparent binding constant of HG380B2 in the pre-stroke state with MTs, and k₃MT is the rate of the MT-activated second isomerization step. By plotting the observed rates of the pre-stroke to post-stroke transition in the presence of MTs against MT concentration, Kk₃MT is estimated as 1.0 μM⁻¹ s⁻¹ (Fig. 5D).

**MT-activated Overall ATP Hydrolysis Is Faster than the MT-activated Pre-stroke to Post-stroke Transition**—The acceleration of the second isomerization by MTs shown in Fig. 5D is expected to result in the MT activation of the pre-stroke to post-stroke transition as well as the MT activation of steadystate ATP hydrolysis, since the second isomerization is the rate-limiting step for both processes (22, 27, 28). To determine whether these two processes are indeed accelerated by MTs in limiting step for both processes (22, 27, 28). To determine whether these two processes are indeed accelerated by MTs in limiting step for both processes (22, 27, 28). To determine whether these two processes are indeed accelerated by MTs, we employed the FRET signal from the GFP-BFP sensors fused to the dynein motor domain as a probe to report tail positions as well as ATPase steps at the primary ATPase site.

The presteady-state kinetics of the change in the FRET signal upon the addition of ATP has revealed that after the rapid formation of the collision complex of the dynein motor domain and ATP, the post-stroke to pre-stroke transition quickly follows at the rate of k₂ = 180 s⁻¹. This rapid isomerization step is expected to correspond to the recovery stroke for priming the next power stroke of the tail. Because the reverse step of this isomerization proceeds with an appreciable rate (k₁₋₂ = 4.0 s⁻¹), this reaction becomes dominant against the forward step at ATP concentrations lower than 2 μM, unlike what is seen for myosin or kinesin whose first isomerization step is virtually irreversible even at low ATP concentrations (33, 34, 37). Therefore, conventional single turnover experiments are not feasible for dynein to determine the rate of the second isomerization step, which is assumed to correspond to the power stroke step (22, 27, 28). It should be noted that this reversible isomerization at a low ATP concentration would be an obstacle for single-molecule experiments such as the observation of single ATP hydrolysis events on dynein because they usually require very low ATP concentrations (~nm) (38, 39).

Here we bypassed this problem by determining the rate of the total flow from the pre-stroke state to the post-stroke state. The apparent rate thus obtained (kobs(pre-post)) is the sum of the rate of the reverse step of the first isomerization (k₋₁) and the rate of the forward step of the second isomerization (k₁). Accordingly, k₋₁ was indirectly estimated to be very small (~0.2 s⁻¹). This slow isomerization rate would be equal to the steady-state ATPase rate at the AAA1 site, whereas the overall rate of ATP hydrolysis by multiple ATPase sites is ~5 s⁻¹ in the absence of MTs (21, 30). Therefore, ATPase sites other than the AAA1 site would be major contributors to overall ATP hydrolysis in the absence of MTs. In fact, a double mutant of the motor domain, P1/P3, in which the P-loops in the AAA1 and AAA3 modules are mutated to block the binding of ATP to the AAA1 and AAA3 sites, still maintains a high ATPase rate (~4 s⁻¹) (21). Thus, it is likely that in the absence of MTs, robust ATP hydrolysis occurs at the AAA2 and/or AAA4 ATPase site(s), whereas the AAA1 and AAA3 sites only slowly hydrolyze ATP. The functional roles of the robust hydrolysis of ATP at the AAA2 and/or AAA4 site(s) in the absence of MTs remain to be resolved.
Kinetics of Dynein Swing Steps

The overall ATPase activity of the motor domain of Dictyostelium cytoplasmic dynein is increased from ∼5 to ∼100 s⁻¹ by saturating levels of MTs (21, 30), unlike the case of axonemal dynein, whose ATPase activity is activated only severalfold with MTs (40). Thus, the issues critical for understanding the molecular mechanism of motor function of cytoplasmic dynein are (i) whether the tail swing probed by FRET is accelerated by MTs as expected from the power stroke model and (ii) which of the multiple ATPase sites in the AAA ring are activated by MTs.

The first issue was addressed by following the FRET signal after suddenly depleting ATP from the mixture of ATP, HG380B2, and MTs. The results showed that MTs actually accelerate the second isomerization but not the first isomerization (Fig. 5), further supporting the notion that the second isomerization detected by the FRET signal corresponds to the tail swing of the power stroke.

The second issue was addressed by comparing the apparent second-order rate constant of the MT activation of the pre-stroke to post-stroke transition and that of steady-state ATP hydrolysis. If the AAA1 site is the primary site responsible for the power stroke of the tail and if it is also the only site where ATP hydrolysis is accelerated by MTs, the apparent second-order rate constants for both processes must be equal because their rates are limited by the same step, i.e. the second isomerization at the AAA1 site. Contrary to the idea that MTs activate only the AAA1 site, the second-order rate constant of the MT-activated ATP hydrolysis is twice that of the MT-activated pre-stroke site(s) other than the primary site may also be activated by MTs. The AAA3 site is a candidate since the ATPase cycle at this site is tightly coupled with that at the primary site (21, 22). Other possibilities must also be considered, however, until the MT-activation of ATP hydrolysis at site(s) other than the primary site is directly confirmed by a probe that can selectively report nucleotide states at each of these additional site(s).

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