Slow ADP-dependent acceleration of microtubule translocation produced by an axonemal dynein

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Abstract Dynein has four nucleotide binding sites, of which the functional significance is unknown except for the single catalytic site. To obtain clues to the function of non-catalytic nucleotide binding, we examined the effect of ADP on the in vitro motility of *Chlamydomonas* inner-arm dynein species 'a'. Upon continuous perfusion with ATP and ADP, microtubules glided on a dynein-coated glass surface with a velocity that gradually increased over a few minutes. The velocity increased faster at higher ADP concentrations. These results suggest that this dynein is activated by nucleotide binding to regulatory site(s) through an extremely slow process.

Key words: Flagellum; Inner-arm dynein; In vitro motility assay; ADP; *Chlamydomonas*

1. Introduction

Dynein is a motor protein involved in many important cellular functions such as axonal transport, cell division, and ciliary and flagellar beating. The beating of cilia and flagella is based on the sliding between outer-doublet microtubules driven by axonemal dyneins, which are attached to the nine outer doublets by their tail regions and generate force through the interaction between their head domains and adjacent doublet tubules. Axonemal dyneins are classified into outer- and inner-arm dyneins, according to their location on the outer doublets (reviewed in [1]). Amino acid sequence analyses of heavy chains have suggested that the dynein head domain, having three extra nucleotide binding motifs (P-loops; P2–P4) besides the P-loop of the catalytic site (P1), belongs to the AAA^+ ATPase family [2–4]. The function of the extra P-loops is unknown, although Mocz and Gibbons [5] have shown that at least some of them can in fact bind ATP and ADP. Several studies have suggested that the binding has some regulatory functions. For example, in vitro motility experiments have demonstrated that the presence of low concentrations of ADP is necessary for some species of *Chlamydomonas* axonemal inner-arm dyneins to generate microtubule translocation [6]. Similarly, the ATPase activity of a species of *Tetrahymena* dynein (possibly an inner-arm dynein) is inhibited when ADP is thoroughly removed from the solution [7]. In experiments with axonemes, ADP greatly enhances ATP-induced sliding disintegration [8] and induces beating in *Chlamydomonas* paralyzed mutants lacking the central pair or radial spokes [9]. Nucleotide conditions may therefore control flagellar beating through modulation of dynein activities.

To learn more about the characteristics of the nucleotide dependence of dynein motility, here we used an in vitro motility system to examine the microtubule translocation produced by *Chlamydomonas* inner-arm dynein species 'a' in the presence of various concentrations of ATP and ADP. The velocity of microtubule translocation in fact appeared to be controlled by the binding of ADP to site(s) other than the catalytic site. In addition, we observed that the apparent binding of ADP is an unexpectedly slow process, proceeding over a time range of a few minutes. Such a slow nucleotide binding process in dynein has not been reported before.

2. Materials and methods

2.1. Preparation of inner-arm dyneins

Dyneins were prepared from the *Chlamydomonas reinhardtii* mutant *oda1* lacking outer-arm dyneins, as described elsewhere [10]. In brief, isolated flagella were demembranated with Nonidet P40 and extracted with 0.6 M KCl. This extract was fractionated into seven inner-arm dynein species by high performance liquid chromatography on a MonoQ column (Fig. 1A).

2.2. In vitro motility assay

In vitro motility assay was carried out essentially according to Kagami and Kamiya [10]. Each species of dynein in HMDE solution (30 mM HEPES, 5 mM $MgSO_4$, 1 mM dithiothreitol, 1 mM EGTA) was applied to a flow chamber (volume: about 7 μl) made of a glass slide, a coverslip, and a pair of spacers. Each dynein sample (0.06 mg/ml) was introduced into the chamber and left for 3 min, followed by introduction of HMDE containing 2 mg/ml bovine serum albumin. After microtubule solution in HMDE was introduced, in vitro translocation was initiated by perfusing the chamber with HMDE containing 10 μM taxol with appropriate concentrations of ATP and ADP. To keep the nucleotide concentrations constant, nucleotide solutions (35 μl) were repeatedly perfused into the chamber every minute. For complete elimination of ADP from the solution, an ATP-regenerating system composed of 5 mM creatine phosphate and creatine kinase (70 U/ml) was included in the perfusion solution. Translocation of microtubules was observed with a dark-field microscope equipped with a SIT camera and recorded on video tapes. Velocities of translocating microtubules were measured with a home-made program [10]. The obtained data were analyzed using a least-squares fitting procedure.

3. Results and discussion

We chose to examine the nucleotide dependence of motility in inner-arm dynein 'a' because the activity of this dynein has been shown to have a strong dependence on ADP [6]. As

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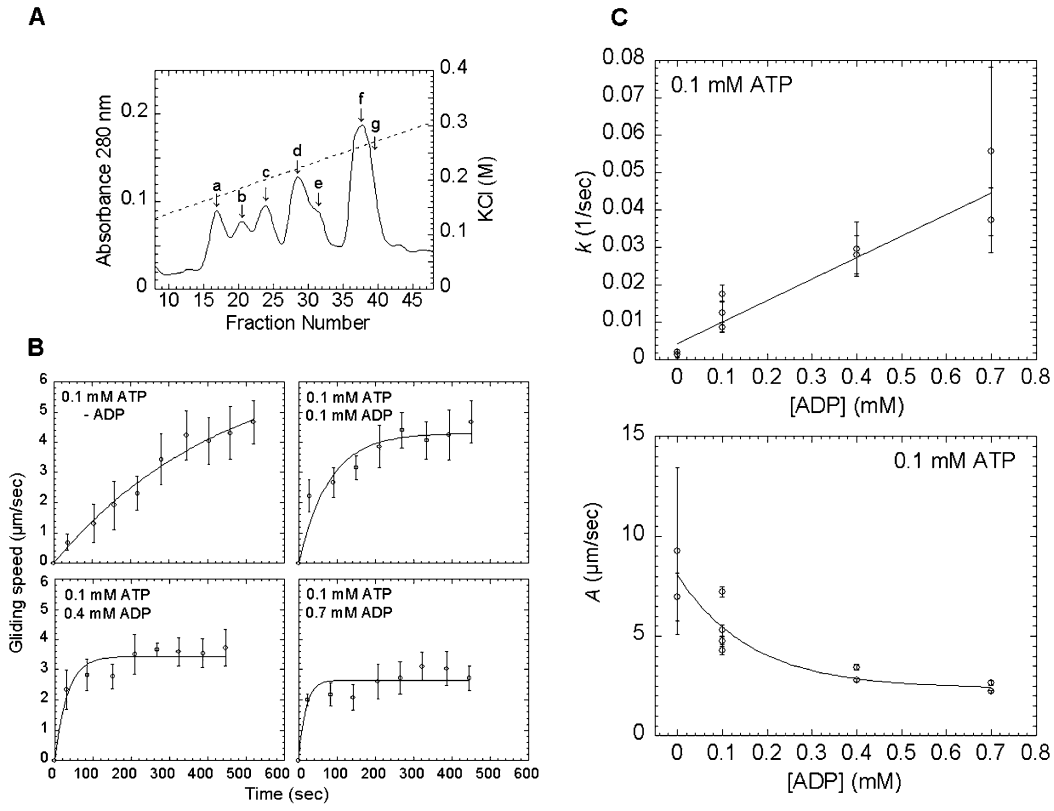

Fig. 1. In vitro motility assay of inner-arm dynein 'a'. A: Separation of inner-arm dyneins a–g by chromatography on a MonoQ anion exchange column. Solid line, absorbance at 280 nm. Broken line, KCl concentration. B: Change in the gliding velocity of microtubules induced by inner-arm dynein a, in the presence of 0.1 mM ATP and various concentrations of ADP. Data in each panel were fitted to the equation: $v = A(1-e^{-kt})$, shown by a solid line. Note that the velocity increases more quickly in the presence of higher concentrations of ADP. C: Two constants of kinetics A and k obtained from the curve fitting used in Fig. 1B. The rate constants, k, were fitted to a straight line. The terminal velocities, A, were fitted to an inverse-proportional function of [ADP], assuming the competition of ATP and ADP at the catalytic site.

reported [6], no microtubule translocation occurred when the sample solution contained 0.1–1 mM ATP and an ATP regeneration system composed of phosphocreatine and creatine kinase, indicating that a trace amount of ADP is prerequisite for its motility (data not shown). When the sample was repeatedly perfused with 0.1 mM ATP without an ATP regeneration system, however, microtubules displayed slow gliding movements. Interestingly, the gliding became faster with time over a range of ~10 min (Fig. 1B). Speculating that the velocity increase might be due to binding of the small amount of ADP produced by dynein ATP hydrolysis to a subset of dynein molecules, we examined the effect of added ADP. As shown in Fig. 1B, the velocity increased faster as the ADP concentration was increased, supporting the idea that the increase in microtubule translocation velocity is brought about by the binding of ADP to some non-catalytic site(s) on dynein. We assume that ADP-bound dynein 'a' generates force to translocate microtubules, whereas ADP-free dynein binds to microtubules without producing active force and acts as a drag. Under this assumption, the velocity of microtubule translocation is proportional to the fraction of the active dynein when the fraction is small. Approximating that the free ADP concentration is equal to the total ADP concentration, we can express the velocity of microtubule translocation (v) at each ADP concentration as $v = A(1-e^{-kt})$, where A denotes the final velocity (a function of ATP and ADP concentrations), and k is the rate constant of the time-dependent change in the ADP-bound dynein concentration; if we express the rate constant of ADP binding to the dynein non-catalytic site by k_1 and that of ADP dissociation by k_{-1}, $k = k_1[\mathrm{ADP}] + k_{-1}$.

Choosing appropriate values for A and k, we found that the experimental data fit this equation reasonably well (Fig. 1B, solid curves). In accordance with the overall rate constant k being a linear function of [ADP] ($k = k_1[\mathrm{ADP}] + k_{-1}$), the k values that matched the experimental data showed a linear dependence on [ADP] (Fig. 1C). From this dependence, k_1 and k_{-1} were determined to be 0.057 ± 0.006 $\mathrm{mM^{-1}\ s^{-1}}$ and 0.004 ± 0.002 $\mathrm{s^{-1}}$, respectively. These values indicate that the activation and deactivation of microtubule translocation velocity by ADP is a slow process that proceeds over a time range of a few minutes. The final velocity attained after prolonged perfusion, in contrast, decreased with the increase in ADP concentration (Fig. 1B). Also from curve fitting, the final velocity of translocation, A, was determined under each condition; it lowers at higher ADP concentrations most likely due to the competition with ATP at the catalytic site (Fig. 1C).

To confirm the slow effect of ADP, dynein in the chamber was first left in the solution containing 0.1 mM ADP alone for certain periods of time, and then perfused with 0.1 mM ATP. As expected, the translocation velocity measured 1 min after the onset of ATP perfusion increased after longer ADP preincubation (Fig. 2, closed circles). However, we also found

that the translocation increased even more when dynein was preincubated with 0.1 mM ADP and 0.1 mM ATP instead of 0.1 mM ADP alone before introduction of microtubules (Fig. 2, open circles). This might indicate some synergistic effect between ATP binding and ADP binding. For example, ADP binding to a non-catalytic site on dynein may be accelerated by ATP binding and/or hydrolysis at the catalytic site.

Next, to examine the reversibility of the ADP effect, the microtubule translocation activity of dynein was fully activated by incubation in 0.1 mM ATP and 0.1 mM ADP for 10 min, followed by washing out of nucleotides by perfusion with HMDE solution containing 10 μM taxol and no nucleotides. After being kept in the nucleotide-free solution, the chamber was again perfused with 0.1 mM ATP and 0.1 mM ADP. The velocity of microtubule translocation 1 min after onset of perfusion was found to decrease after longer nucleotide-free incubation (Fig. 3). In each case, the velocity of microtubule translocation gradually increased as ATP/ADP perfusion was continued (not shown). These results support that, in nucleotide-free conditions, the ADP bound to the non-catalytic site of dynein is slowly released and dynein is concomitantly and reversibly inactivated. In this experiment, the dissociation constant of ADP was estimated to be $0.009 \pm 0.002\ s^{-1}$.

The above results indicate that the slow increase in microtubule translocation velocity can be explained by a model that postulates activation of dynein by slow binding of ADP to some of the regulatory nucleotide binding sites (P2–P4). The site is most likely other than the catalytic site (P1) since the apparent binding rate constant is much slower than that of ATP hydrolysis. In view of the recent reports that nucleotide binding to the P3 site is particularly important for the motor function of cytoplasmic dynein [11,12], ADP binding to the P3 site may also be important for the function of the axonemal dynein used in this study. Whichever the binding site is, it must cause different effects depending on what nucleotide it binds, since ADP but not ATP can accelerate the increase in microtubule gliding velocity (Fig. 1B).

Although whether dynein 'a' actually binds ADP at its regulatory site very slowly awaits further experiments that directly detect nucleotide binding, it is certain that dynein activity can be regulated by an extremely slow process

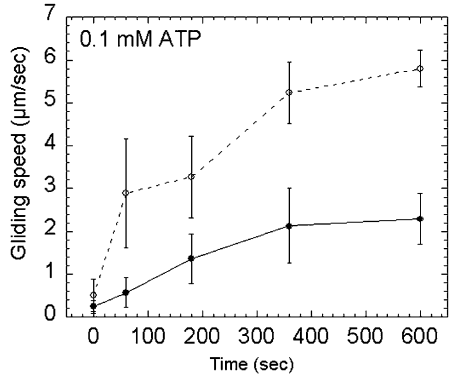

Fig. 2. Slow activation of dynein activity in the absence of microtubules. Dynein was incubated with 0.1 mM ADP alone (closed circles), or 0.1 mM ATP and 0.1 mM ADP (open circles) for appropriate periods before microtubules and 0.1 mM ATP were introduced. The velocity of microtubule translocation was measured 1 min after the introduction of microtubules/ATP.

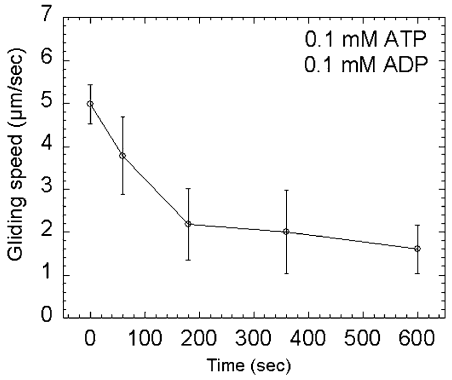

Fig. 3. Reversibility of ADP-induced activation. The motile activity of dynein was first fully activated by incubation in the presence of 0.1 mM ATP and 0.1 mM ADP for 10 min. The sample was then perfused with a nucleotide-free solution for various periods, followed by perfusion with 0.1 mM ATP and 0.1 mM ADP. The gliding velocity of microtubules was measured 1 min after the introduction of microtubules/ATP.

dependent on nucleotides. This is the first time that such a slow phenomenon has been found in dynein. Other motor proteins have also been shown to display extremely slow processes; for example, the ADP dissociation rate constant of kinesin is as low as $0.009\ s^{-1}$ [13] and the product release rate constant of myosin II is $0.05\ s^{-1}$ [14]. These slow processes are, however, greatly accelerated when the motor proteins interact with microtubules or actin filaments. The slow process found in this study differs from those observed in other motor proteins since the slow activation of dynein activity occurs while dynein is interacting with microtubules. The slow regulation by nucleotide binding as inferred from the present study may be a property shared by other dyneins; in fact, in our preliminary experiments, we observed inner-arm dynein 'c' also displays a slow increase in translocation velocity after ATP perfusion (data not shown). We do not yet understand the physiological importance of the ADP-dependent modulation of dynein activity but it may have a function to regulate axonemal motility according to the average nucleotide conditions within the axoneme. The observed slow change also indicates that we need caution in interpreting the results of in vitro motility assay experiments using dynein.

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