Systematic Comparison of in Vitro Motile Properties between Chlamydomonas Wild-type and Mutant Outer Arm Dyneins Each Lacking One of the Three Heavy Chains*

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Outer arm dynein (OAD) of cilia and flagella contains two or three distinct heavy chains, each having a motor function. To elucidate their functional difference, we compared the in vitro motile properties of Chlamydomonas wild-type OAD containing the α, β, and γ heavy chains and three kinds of mutant OADs, each lacking one of the three heavy chains. For systematic comparison, a method was developed to introduce a biotin tag into a subunit, LC2, one of the three heavy chains. For systematic comparison, a method was developed to introduce a biotin tag into a subunit, LC2, which served as the specific anchoring site on an avidin-coated glass surface. Wild-type OAD displayed microtubule gliding in the presence of ATP and ADP, with a maximal velocity of 5.0 μm/s, which is approximately 1/4 of the microtubule sliding velocity in the axoneme. The duty ratio was estimated to be as low as 0.08. The absence of the β heavy chain lowered both the gliding velocity and ATPase activity, whereas the absence of the γ heavy chain increased both activities. Strikingly, the absence of the α heavy chain lowered the gliding velocity but increased the ATPase activity. Thus, the three heavy chains are likely to play distinct roles and regulate each other to achieve coordinated force production.

The rhythmic beating of eukaryotic cilia and flagella is produced by a regulated interaction between axonemal dyneins and outer doublet microtubules (MTs). The axonemal dyneins are classified into inner arm dyneins (IADs) and an outer arm dynein (OAD) according to their positions on the outer doublet. Each dynein is an ATPase complex composed of one to three heavy chains (HCs) and several smaller subunits. The HCs are composed of a head domain that produces a sliding force through an ATP-sensitive interaction with MTs and a tail domain that is stably fixed to the outer doublet A-tubule. In many organisms, OAD exists as a single large molecular complex, whereas the IADs exist as multiple, smaller complexes. Studies using Chlamydomonas reinhardtii mutants showed that IADs are important for the generation of a proper flagellar waveform, whereas OAD is important for the generation of high beat frequency (1, 2), suggesting that different dynein species have distinct functions. Functional diversity is also found among the multiple HCs in a single OAD complex; mutants that lack any one of the three OAD HCs (α, β, and γ), as well as mutants lacking the entire OAD, display reduced motility, but the degree of the motility defects varies depending on the missing HC (3–5). Thus, each HC apparently has a distinct role in OAD function. An important challenge is to elucidate how different HCs share their roles in the OAD complex to produce the overall movement.

In accordance with in vivo observations, assays of in vitro MT gliding on dynein-coated surfaces have revealed motility differences among different outer arm HCs. In sea urchin OAD, which contains two HCs (α and β), a partial assembly that contains the β HC and an intermediate chain is capable of translocating MTs, whereas the α HC is not (6–8). In Chlamydomonas OAD, the velocity of in vitro MT gliding induced by partial OAD assemblies differs greatly between those containing both α and β HCs, those containing only the β HC, and those containing only the γ HC (9). However, these studies were based on experiments in which OAD complexes were attached to a glass surface in an uncontrolled manner; they were allowed to spontaneously adsorb on the surface during perfusion of an observation chamber with a solution containing the OAD complex. This is a problem because the dynein HCs do not necessarily attach to the surface by the tail domain, and some fraction may attach by the motor domain; inversely attached HCs may stick out their tail, which will interact with the MT and interfere with its movement. Therefore, the observed difference in motility between different OAD subparticles could have been caused by a difference in their manner of attachment to the glass surface rather than by a difference in their motor activity.

To overcome this problem, in this study we developed a method for specifically fixing dynein complexes to a glass slide using a novel labeling method that takes advantage of a biotin-avidin interaction. Motility assays with this improved system enabled us to characterize the in vitro properties of Chlamydomonas OAD and compare the performance of wild-type OAD and OADs lacking each of the three HCs. Our data revealed complex interactions between HCs that have not been observed before.

**Experimental Procedures**

Strains and Media—C. reinhardtii mutants used in this study were oda12-1nit1 (a double mutant between oda12-1, which is
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a null mutant of the genes of two light chains LC2 and LC10 and lacks the entire outer arm, and the mutant nit1, which is deficient in nitrate reductase; this double mutant will be referred to as oda12 (10, 11), oda11 (lacking the α HC) (3), oda4-s7 (lacking the motor domain of the β HC) (4), and oda2-t (lacking the motor domain of the γ HC) (5). The cells were grown in liquid Tris-acetate-phosphate medium with aeration on a 12 h of light/12 h of dark cycle. When necessary, the cells were grown on solid medium containing 1.5% agar and supplemented with zeocyn.

Construction and Expression of the LC2-BCCP Fusion Protein—For use in an avidin-biotin labeling system, full-length Chlamydomonas LC2 cDNA was modified to carry a biotin carboxyl carrier protein (BCCP) (12) at its C terminus. For efficient expression in Chlamydomonas, cDNA of a minimal domain required for biotinylation was subcloned from the cDNA of the Chlamydomonas acetyl-CoA carboxylase biotin carboxyl carrier protein subunit (C590042 GI Chlamydomonas genome data base v2.0) and fused to the LC2 cDNA. This fusion construct (LC2-BCCP) was inserted into the pGenD vector (13) and introduced by electroporation into oda12. A strain expressing native OAD containing the LC2-BCCP fusion protein (wt-lc2-bccp) was obtained by screening transformed cells for growth in the Tris-acetate-phosphate medium containing zeocyn, fast swimming, and the presence of LC2-BCCP as detected by Western blotting. Flagellar beat frequency was assessed from the power spectrum from a population of swimming cells (14).

Mutants lacking one of the three OAD HCs (oda11, oda4-s7, and oda2-t) were crossed with wt-lc2-bccp to obtain strains expressing partially defective outer arms with LC2-BCCP. The resultant strains will be referred to as oda11-lc2-bccp, oda4-s7-lc2-bccp, and oda2-t-lc2-bccp, respectively.

Dynein Purification—Flagellar axonemes were isolated and demembranated by standard methods (15). Dyneins extracted from axonemes with 0.6 M KCl in HMDE solution (30 mM HEPES-NaOH, 5 mM MgSO4, 1 mM dithiothreitol, and 1 mM EGTA, pH 7.4) were first diluted 5-fold with HMDE solution to lower the salt concentration, clarified by centrifugation at 72,000 × g for 20 min, applied onto a UnoQ anion exchange column (Bio-Rad), and eluted with a linear gradient of 150–400 mM KCl in HMDE solution. Peak fractions containing OAD with LC2-BCCP were pooled, and the protein concentration was determined by the method of Bradford (16). Aliquots of pooled dynein fractions were stored in liquid nitrogen after the addition of sucrose and bovine serum albumin to final concentrations of 30% and 0.1 mg/ml, respectively.

MT Preparation—Porcine brain tubulin was purified by two cycles of assembly/disassembly and chromatography on a phosphocellulose column (P11; Whatman) (17). MTs were polymerized from 2 mg/ml tubulin in the presence of 1 mM GTP at 37 °C for 30 min and were stabilized with 40 μM paclitaxel (T1912; Sigma). For fluorescence microscopy, MTs were labeled with tetramethylrhodamine. Polarity-marked MTs were prepared using a 2:3 ratio of N-ethylmaleimide-treated to N-ethylmaleimide-untreated tubulin (total tubulin concentration, 2 mg/ml) and fragmented axonemes (18).

In Vitro Motility Assays—OAD association with MTs was examined under a fluorescence microscope (IX-71; Olympus). Biotinylated OAD was labeled with quantum dot-streptavidin (Qdot; Q10121MP; Invitrogen). ATP-dependent dissociation of Qdot-OAD from rhodamine-labeled MTs was induced by applying ATP by photolysis of 50 μM caged ATP with an ultraviolet laser and observed using a conventional fluorescence microscope with a 100×/1.25 NA objective. The movement of OADs along MTs was observed under evanescent illumination through a 60×/1.45 NA objective. Fluorescent images were captured with a cooled electron multiplier charge coupled device at 33-ms intervals. The centroid of the Qdot image was determined by fitting the image density distribution to a two-dimensional Gaussian function (19).

A MT gliding assay was performed using a flow chamber (volume, ~5 μl) made of a glass slide (S-1215; Matsunami) and a coverslip (18 × 18 mm; Matsunami) with two spliers of polyeester film (Lumirror 25T60; Toray) as spacers. The chamber was sequentially coated with 2 mg/ml biotinamidocaproyl BSA (A6043; Sigma) and 1 mg/ml streptavidin (19–11644; Wako) and washed with 5 mg/ml BSA (A7906; Sigma) in HMDE solution. Purified dynein (20 nm) was then perfused into the chamber so as to allow the molecules to be fixed on the glass surface through biotin-avidin linkage. Following incubation for 3 min with HMDE solution containing 1 mg/ml BSA, 1 mM ADP, and 1 mM ATP, the chamber was filled with paclitaxel-stabilized MTs (~5 μg/ml), 1 mg/ml BSA, 1 mM ADP, and 1 mM ATP in HMDE solution. When the effect of ADP preincubation was examined, MTs in HMDE were introduced into the chamber together with 1 mM ATP after the dynein-coated chamber had been incubated with 0–3 mM of ADP. The surface density of dynein molecules was determined by quantitative Western blotting. Dynein on the glass surface was removed and retrieved by introducing sample buffer (50 mM Tris-HCl, 2% SDS, 5% β-mercaptoethanol, 12% glycerol, pH 7.4), separated by SDS-PAGE, and immunoblotted using anti-LC2 antibody. Through this procedure, we estimated that ~40% of the dynein introduced into the chamber was fixed to the glass surface. MT gliding was observed by dark field microscopy using a BX50 microscope (Olympus) equipped with a 40×/0.85 NA objective, a dark field condenser, and a 100 W mercury arc light source. The images were recorded with an silicon intensified target camera (Hamamatsu Photonics), digitized, and analyzed on a personal computer. MT gliding velocities were measured using images of MTs that exhibited continuous movements over more than 10 μm. MTs longer than 10 μm were chosen except when the dependence on MT length was examined. The duty ratio, the time fraction of an ATPase cycle wherein an OAD molecule is attached to a MT and is engaged in force production, was determined from the dependence of MT gliding velocity on the OAD surface density (20–22).

Steady-state ATPase Assays—ATPase rate was measured using an EnzChek phosphate assay kit (E6646; Invitrogen). Purified dynein (5–10 nM) and ATP in HMDE solution were supplemented with the reaction mixture to give a total volume of 100 μl, and the phosphate released in the solution was continuously monitored at 360 nm for 5 min at 25 °C. Each measurement was repeated at least three times with different prep-
Electrophoresis and Western Blot Analysis—The dynein HC composition was analyzed by SDS-PAGE with a 3–5% polyacrylamide gradient and 3–8 M urea gradient (22). Biotinylation of LC2-BCCP was detected with a Vectastain ABC kit (Vector Laboratories). To detect LC2, the blots were probed with affinity-purified anti-LC2 polyclonal antibody R5391 (23), which was a gift from Dr. Steve King (University of Connecticut Health Center).

RESULTS

Production of an OAD Complex Containing a Biotinylated Light Chain—For reliable in vitro motility assays, isolated dyneins must be attached to a solid surface by the tails, whereas their head domains are kept free to interact with MTs (Fig. 1 and see below). To achieve this condition, we devised a method to specifically label light chain LC2, which is attached to the OAD tail region (24). This light chain is essential for proper OAD assembly, as evidenced by the fact that an LC2/LC10 null mutant, oda12, does not assemble functional OAD (10, 26).

Although oda12 lacks both LC2 and LC10, its motility defects can be rescued by transformation with a genomic fragment containing only the wild-type LC2 gene (10, 11). We constructed a cDNA that encodes an LC2-BCCP fusion protein and used it to transform oda12. The transformed cells expressed LC2-BCCP and recovered almost wild-type motility (Fig. 2A).

FIGURE 1. Schematic drawing of the in vitro motility assay system used. Biotinylated OAD was fixed on a glass surface via streptavidin, which is specifically bound to the biotinamidocaproyl BSA attached to the glass surface.

FIGURE 2. Construction of the OAD complex containing LC2-BCCP: A, flagellar beat frequency was assessed from the power spectra of vibration signal in a population of cells (14). Gray, wild type; black, oda12 and oda12-expressing LC2-BCCP. Arrows, peaks of the spectra, which give the estimates of the average beat frequency. Cells expressing LC2-BCCP recovered the wild-type beat frequency, indicating production of functional OAD. The swimming velocity of oda12 expressing LC2-BCCP was 128 ± 9 μm/s, whereas that of wild type was 141 ± 14 μm/s. When the former cells were left in a Petri dish illuminated sideways, the cells gathered around the side near the light source, as did wild-type cells; hence both are positively phototactic. When the former cells were observed with a dark field microscope with red light and suddenly illuminated with strong white light, they transiently swam backwards, as did wild-type cells; hence both can display a photophobic response. B, chromatographic separation of OAD from wt-lc2-bccp. High salt extract from wt-lc2-bccp axonemes was fractionated on a UnoQ column after desalting. Peaks b–g indicate inner arm subspecies; peaks γ, αβγ, and αβ indicate the OAD and its dissociated subparticles. Inset, SDS-PAGE showing the dynein HC region. C, electrophoresis and Western blot analysis of purified OAD. Left two lanes, electrophoretic analysis of purified OADs from wild-type (expressing LC2) and wt-lc2-bccp (expressing LC2-BCCP) cells. Both contain three HCs (top panel; separated on a 3–5% acrylamide and 3–8 M urea gradient gel) and several light chains (bottom panel; separated on a 12% acrylamide gel). The LC2 band, ~16 kDa, is present in the wild-type lane, whereas the LC2-BCCP band, ~30 kDa, is present in the wt-lc2-bccp lane. Right two lanes, the LC2-BCCP band in wt-lc2-bccp detected by an anti-biotin antibody.
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indicating that LC2-BCCP functions as well as native LC2. Somewhat surprisingly, they showed apparently normal phototaxis and photophobic responses. Thus, it is not clear what defects have been caused by the absence of LC10 in this strain. The OAD in the transformed cells could be purified from axonemes in the same manner as from wild-type axonemes, and the amount of OAD obtained from the transformants was similar to that obtained from wild-type cells. The isolated OAD contained biotinylated LC2-BCCP (∼30 kDa) instead of wild-type LC2 (∼16 kDa), as detected by Western blot analysis (Fig. 2C).

Strains that have OADs containing LC2-BCCP and lacking a particular HC or the motor domain of a particular HC were produced by crossing the LC2-BCCP-expressing oda12 cells (wt-lc2-bccp) with the mutants oda11 (lacking the α HC), oda4-s7 (lacking the motor domain of the β HC), or oda2-t (lacking the motor domain of the γ HC). Production of the expected mutant was confirmed by examining the composition of the HCs and biotinylation of the LC2 protein in the axonemes of the daughter cells (Fig. 3A). These dyneins were also purified and used for gliding assays. (The complex lacking γ HC and containing α and β HCs was more conveniently prepared from wt-lc2-bccp cells, because this complex was obtained as a partially dissociated by-product of the αβγ complex (Fig. 2B, lane αβ)). Thus, one kind of three-headed and three kinds of two-headed OADs containing biotinylated LC2-BCCP became available (Fig. 3, B and C). Hereafter, we refer to the wild-type OAD complex containing LC2-BCCP as αβγ, and the complex that lacks the α HC, the motor domain of the β HC, and the motor domain of the γ HC as βγ, αγ, and αβ, respectively.

Qdot-Avidin-labeled OAD Binds to MTs in an ATP-sensitive Manner—We labeled the biotinylated OADs with Qdot and examined their binding to MTs with a total internal reflection fluorescence microscope. In the absence of ATP, the three-headed OAD-Qdot associated with MTs in an ATP-sensitive manner (Fig. 4A, Movies S1 and S2). This indicates that Qdot-conjugated OAD interacted with MTs through the ATP-sensitive MT-binding sites. Moreover, we observed that a small number of OADs remained attached to MTs after photoreleasing of 4.5 µM ATP and moved along them (Fig. 4, B and C and supplemental Movie S2). However, the movement was diffusive and not unidirectional, suggesting that the single molecules of OAD cannot take stable, successive steps upon encountering a MT.

Stable MT Gliding on a Glass Surface Coated with Biotinylated OAD—For observation of MT gliding on a glass surface, an avidin-coated chamber was perfused with the biotinylated OADs of various HC compositions, followed by introduction of MTs, ATP, and ADP (see “Experimental Procedures”). ADP
was added because we found that stable gliding required the presence of ADP, as shown below. MTs displayed gliding over glass surfaces coated with any biotinylated-OAD complex constructed in this study (Fig. 1). In addition, the velocity of gliding induced by αβγ, 6.8 ± 1.3 μm/s in our system, was higher than the previously reported value of 4.6 ± 1.0 μm/s (9), suggesting that specific anchoring via the biotin-avidin interaction is effective for producing efficient MT gliding. The specificity of the binding of OAD-biotin to avidin was confirmed by an assay in which the avidin-coated chamber was pretreated with 5 mM biotin; MTs did not bind to the glass surface of a chamber that had been sequentially perfused with biotin and biotinylated OADs. This observation confirmed that the OADs were fixed on the glass surface selectively via the avidin-bitoin interaction.

**Dependence of Gliding Velocity on ATP Concentration and OAD Surface Density**—Using this in vitro assay system, we first characterized the motile properties of three-headed OADs (αβγ). With a given OAD concentration (20 nM), the MT gliding velocity showed a hyperbolic dependence on ATP concentration with a maximal velocity of 5.0 ± 0.2 μm/s and an apparent Michaelis constant of 67 ± 10 μM. This maximal velocity is approximately ¼ of the maximal MT sliding velocity in disintegrating wild-type axonemes (27). The velocity was also dependent on the OAD surface density (Fig. 5B). This indicates that OAD is a low duty ratio motor that spends most of its ATPase cycle detached from MTs. Curve fitting (20–22) yielded a duty ratio estimate for αβγ of 0.08, which is smaller than that of inner arm dyneins studied thus far: e.g. 0.14 for species c (21) and 0.63 for species f (28). As expected from the low duty ratio, longer MTs, which interact with greater numbers of OAD molecules, were found to display higher speeds (Fig. 5C). From the MT length, the OAD surface density, and the OAD molecular geometry, we estimated that approximately two OAD molecules interacted with a MT per micrometer in a typical experiment (Fig. 5C). This density is ~20-fold lower than the density of OAD molecules attached to the outer doublet in the axoneme, i.e. 40 OADs/micrometer.

**ADP Incubation of OAD Enhances and Stabilizes MT Gliding**—Previous studies have shown that MT gliding by some species of axonemal dynein accelerates when the buffer contains ADP in addition to ATP or when dynein has been preincubated with ADP (29–32). This phenomenon is thought to be caused by ADP binding to a regulatory nucleotide-binding site(s) of HCs. To determine whether this kind of nucleotide dependence is also present in *Chlamydomonas* OAD, we examined the effect of ADP preincubation. An OAD-coated glass surface was first left in contact with various concentrations of ADP (0–3 mM) for 3 min or with 1 mM ADP for various time periods, and then MT gliding was initiated by introducing MTs and 1 mM ATP. Preincubation of OAD with ADP increased both the velocity and duration of MT gliding (supplemental Fig. S1). The gliding velocity reached a plateau at ~1 mM ADP when the incubation time was fixed at 3 min (Fig. 6A), and at ~3 min when the ADP concentration was fixed at 1 mM (Fig. 6B). Without ADP preincubation, MTs did not show smooth unidirectional gliding but displayed irregular back-and-forth movements immediately after the introduction of MTs and ATP into the chamber. These observations suggest that OAD is activated by ADP through a slow process, similar to other kinds of dyneins studied previously (31, 32). Thus, ADP regulation of *in vitro* dynein activity, first observed in inner arm dyneins (29), is a feature shared by *Chlamydomonas* OAD.

Two-headed OADs of any Combination of HCs Can Translocate MTs, but at Different Velocities—The motile properties of two-headed OAD complexes were compared at constant OAD densities and nucleotide concentrations. Observations using polarity-marked MTs demonstrated that all OAD complexes
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exhibited minus-end-directed motility (data not shown). As expected from the swimming velocity difference in mutant cells, each kind of two-headed OAD exhibited a distinct MT gliding velocity. To our surprise, $\alpha\beta$ displayed faster MT gliding than $\alpha\gamma$. In contrast, $\alpha\gamma$ and $\beta\gamma$ displayed slower velocities than $\alpha\beta\gamma$. The movement displayed by $\beta\gamma$ was particularly slow and appeared unstable; MTs often dislocated sideways during gliding (Fig. 7A and Table 1).

The ATPase Activity of OAD Is Not Directly Coupled with Its MT Gliding Activity—Fig. 7B shows the rates of ATP hydrolysis by the four kinds of OADs as a function of ATP concentration. Like the MT gliding activity, the ATPase rate clearly differed from one OAD species to another. Surprisingly, $\beta\gamma$ as well as $\alpha\beta$ displayed higher ATPase activities than $\alpha\beta\gamma$. In contrast, $\alpha\gamma$ displayed much lower activity than $\alpha\beta\gamma$. Therefore, the ATPase rates of $\alpha\beta$ and $\alpha\gamma$, but not that of $\beta\gamma$, parallel the velocities of MT gliding they produce. The $\beta\gamma$ complex showed higher ATPase activity and lower MT gliding velocity than $\alpha\beta\gamma$ (Table 1).

For understanding the mechano-chemical coupling in these dyneins, it is important to examine their MT-activated ATPase. However, the *Chlamydomonas* outer arm dynein did not show significant ATPase activation by 1–10 mg/ml MTs (supplemental Fig. S2). This is not surprising because a previous study with the *Tetrahymena* outer arm dynein showed that ATPase activation was observed only with extremely high concentrations (up to 50 mg/ml) of MTs (33). In sea urchin also, inner arm dyneins, but not outer arm dynein, were reported to display MT-dependent ATPase activation (34). We therefore indirectly evaluated the MT-activated OAD ATPase using axonemal ATPase values, following the previous reports that axonemal ATPase is activated by outer doublet MTs (35–37). The MT-activated OAD ATPase for each species of OAD was estimated by the difference in ATPase between axonemes with OAD and *oda2* axonemes without OAD (Table 1). The ATPase activities thus estimated for $\alpha\beta\gamma$, $\alpha\gamma$, and $\alpha\beta$ roughly parallel their in vitro gliding velocity. As in the basal ATPase, $\alpha\beta$ displays higher ATPase than $\alpha\beta\gamma$. However, the ATPase activity of $\beta\gamma$ in the axoneme is unusually high for its poor in vitro motility.

$\alpha$ HC Is Required for Efficient Mechanochemical Energy Conversion—The peculiar properties of $\beta\gamma$ prompted us to examine its in vitro motility in more detail. Fig. 8 compares the time courses of the change in MT gliding velocities induced by $\alpha\beta\gamma$ and $\beta\gamma$. Compared with $\alpha\beta\gamma$, $\beta\gamma$ showed not only a lower MT gliding velocity but a shorter duration, which may be due to faster consumption of ATP. The lower gliding velocity was not due to the degeneration of the motor protein because reincubation with ATP recovered the original MT gliding velocity.

These results suggest that $\beta\gamma$ is unable to efficiently convert the energy of ATP hydrolysis into mechanical work. We speculated that $\alpha$ HC may facilitate MT gliding by increasing the affinity of OAD for MTs. To test this idea, we examined the effect of methylcellulose addition to the gliding assay solution. Methylcellulose is an inert polymer frequently used in motility assays to reduce Brownian motion of actin filaments or MTs and thereby facilitate their binding to the motor proteins on the glass surface (20–22). However, we were unable to find any significant difference in velocity with and without methylcellulose (Fig. 8), although MT gliding became apparently smoother and more stable in the presence of methylcellulose. This result suggests that the $\alpha$ HC accelerates MT gliding not simply by promoting binding between the MT and OAD but possibly by changing the overall efficiency of OAD as a mechanochemical transducer.

**DISCUSSION**

Motility Measurements in OADs with Different Combinations of HCs—In this study, we compared the in vitro motility properties of *Chlamydomonas* wild-type and mutant OADs each lacking one of the three HCs. Such a systematic comparison of OAD, one of the best characterized axonemal dyneins in all organisms, has not been carried out previously. It was made possible by recent technical advances in the experimental system. First was the development of a method for labeling OAD at a specific site. This was achieved by expressing an OAD light chain fused with a BCCP protein, which is specifically biotinylated by endogenous biotinylation enzymes. BCCP-mediated biotiny-
Gliding velocity and ATPase activity of wild-type and mutant OADs

The data represent the means ± S.D. The ATPase data are averaged from more than three measurements with different preparations.

| OAD type | MT gliding velocitya | Basal ATPase | Swimming velocity of live cellsb | Axonemal OAD ATPasec |
|----------|----------------------|--------------|--------------------------------|----------------------|
|          | µm s⁻¹ |                  | µM |                           | µM s⁻¹ |                      | (µg axoneme)⁻¹ min⁻¹ |
| αβγ      | 4.8 ± 1.1 (n = 192) | 22 ± 1 | 17 ± 2 | 161.6 ± 9.2  | 1.24 ± 0.08 |
| βγ       | 0.87 ± 0.34 (n = 106) | 36 ± 1 | 26 ± 3 | 120.4 ± 13.0 | 0.95 ± 0.10 |
| αγ       | 2.3 ± 0.65 (n = 130) | 12 ± 1 | 5.8 ± 0.7 | 59.7 ± 7.4  | 0.25 ± 0.06 |
| αβ       | 6.8 ± 1.3 (n = 126) | 51 ± 4 | 27 ± 9 | 87.8 ± 9.4  | 1.68 ± 0.30 |

a Measured at 1 mM ATP. The temperature was 25°C.
b Swimming velocity of wild type, oda1 (with the βγ OAD), oda4-s7 (αγ OAD), and oda2-t (αβ OAD) cells. The swimming velocity of the oda2 mutant cells lacking the entire outer arm is 50.6 ± 9.6 µm/s. The data are from Liu et al. (5).
c The ATPase activities in the wild type, oda1, oda4-s7, and oda2-t axonemes subtracted by the activity in the oda2 axoneme lacking the entire OAD. The data are from Liu et al. (5).

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Requirement of ADP for OAD Function—Our results clearly showed that ADP is required for efficient MT gliding by *Chlamydomonas* OAD. Previous studies have shown that MT sliding in the flagellar and ciliary axonemes of various organisms is inhibited by a high concentration of ATP and apparently is activated by ADP (48–52). Moreover, isolated inner arm dyneins have been shown to display higher MT gliding velocity (29) and ATPase activity (30) when ADP is present along with ATP. These phenomena have been thought to be caused by ADP binding to noncatalytic nucleotide-binding sites of dynein HC. Both the activation induced by ADP and the deactivation by the removal of ADP are slow processes that proceed in the time scale of minutes (31) caused by the slow rate constants of ADP binding to and dissociating from the regulatory sites (32). Our observation that preincubation of OAD with ADP resulted in faster MT gliding with a long duration suggests that motility activation by ADP binding is a common feature of many kinds of axonemal dyneins. However, the true mechanism underlying this phenomenon remains to be elucidated.

A previous study has shown that the axoneme of nonmotile *Chlamydomonas* mutants ("paralyzed flagella" mutants) that lack either the central pair MTs (pf18) or the radial spokes (pf14) can be reactivated to beat by the simultaneous presence of ATP and ADP (53). Importantly, this ADP-induced beating apparently requires the presence of OAD, because the axonemes from double mutants lacking OAD, oda1pf18, or *oda1pf14* do not beat even in the presence of ADP (46). These observations led us to propose that the paralyzed flagella of these mutants can beat in the presence of ADP because OAD is

for Ncd (44). Motor proteins with a low duty ratio are common to motile systems in which many motor proteins are involved in force generation at a time, like the muscle actomyosin system (see Ref. 45 for review). On the other hand, the duty ratio of some IADs has been estimated to be significantly larger than that of the OAD measured here. This difference might reflect the functional difference between IADs and OAD. Namely, IADs are important for the generation of a proper flagellar waveform, whereas OAD is important for the generation of high beat frequency (1, 2). In addition, IADs are thought to be directly regulated by central pair/radial spokes, whereas the OAD is not (46, 47). Thus, we may expect that IADs and OAD are designed to interact with MTs in strikingly different manners.

Motile Properties of Wild-type OAD—The MT gliding velocities measured at different OAD densities indicated that the duty ratio of OAD is as low as 0.08 (Fig. 5B). A low OAD duty ratio is consistent with the observation that individual Qdot-labeled OAD molecules were unable to move processively along MTs. The estimated duty ratio is similar to those reported for other nonprocessive motors, e.g. 0.05 for myosin II (43) and 0.1 for Ncd (44).
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activated and that activated OAD can override the inhibitory effects caused by the absence of the central pair or the radial spokes (46, 47). However, no evidence had been obtained for the postulated ADP activation of OAD. The present study has provided the first evidence that OAD is, in fact, activated by ADP.

Distinct Properties of the Three HCs—Comparison of the MT gliding velocity and ATPase activity between the two- and three-headed OADs revealed distinct properties of each HC. The αβ complex showed higher ATPase activity and MT gliding velocity than αβγ, suggesting that the γ HC in the αβγ complex is lowering both the ATPase and MT gliding activities of αβ. This finding is consistent with previous reports that the ATPase activity of αβ decreases when combined with γ HC (54) and that the ATPase activity of oda2-t axonemes with an OAD lacking α HC is greater than that of wild-type axonemes (5). Thus, the γ HC apparently functions as a brake in MT gliding. In contrast to αβ, αγ showed much lower ATPase activity and gliding velocity than αβγ. Thus, the β HC might well be regarded as the main force generator coupled with ATP hydrolysis. Finally, most unexpectedly, βγ showed high ATPase activity yet produced very slow MT gliding. Because the addition of methylcellulose did not accelerate the MT gliding, we surmise that the lack of the α HC greatly decreases the velocity not simply by impairing the interaction between the OAD and the MT, but possibly by changing the properties of the OAD-MT interaction. Consistent with this finding, a previous study has shown that association of α HC decreases the ATPase activity of β HC (40). The higher ATPase rate of βγ over αβγ suggests that the α HC functions to suppress motility-uncoupled ATP hydrolysis by OAD. At the same time, βγ has higher apparent MT-activated ATPase in the axoneme than αγ, whereas it displays slower MT gliding in vitro (Table 1), suggesting that the α HC is important also for production of directional movements.

Comparison with OAD Properties in the Axoneme—Fast MT gliding by αβ and extremely slow gliding by βγ are inconsistent with the motility of mutant axonemes that have these OADs; the oda2-t mutant having αβ OAD displays much poorer motility than wild type, and the oda11 mutant having βγ OAD displays fairly good motility compared with other oda mutants (Table 1). These observations suggest that the properties of OAD in the axoneme differ from those of OAD in vitro. There are several factors we must consider. First, in the axoneme, OAD is anchored to the A-tubule of the doublet through the association of multiple light/intermediate chains with tubulin (26) and is arranged unidirectionally, whereas in vitro it is arranged randomly on the glass surface through an avidin-biotin link. Second, in the axoneme, the OAD is attached to the doublet at 24-nm intervals (~40 OADs are attached per micrometer), and adjacent OADs physically interact with one another (56–59). In contrast, only two or three OADs are attached to the MT per micrometer in vitro (Fig. 5C), precluding an interaction between OAD particles. The interaction between adjacent OADs may modulate the activity of individual OADs. Finally, MT sliding in the axoneme is based on interactions between the MT, OAD, IADs, and other axonemal components, whereas MT gliding in vitro is based only on the MT-OAD interaction. Some or all of these differences may well have caused the observed velocity difference between in vitro gliding and axonemal disintegration.

We surmise that an understanding of the origin of the observed difference is the key to understanding the mechanism of flagellar beating, as well as the functional importance of the ordered arrangement of OADs in the axoneme. A limitation in correlating the OAD properties in vitro and those in the axoneme is that we do not have information regarding the force generated in vitro. Force measurements may provide important clues for the exact roles of individual OAD HCs.

The present study thus revealed a significant difference between OAD function in an in vitro system and in the axoneme. In addition, it clearly showed that the three OAD HCs have strikingly different properties and that the overall behavior of the OAD complex is not simply the sum of the activity of each HC. The observation that some two-headed OADs have higher ATPase activities than the wild-type three-headed OAD but display slower MT gliding suggests the importance of inter-HC interactions for efficient OAD functioning. The inter-HC interaction in OAD seems to be more complex than in cytoplasmic dynein or kinesin in which two equivalent HCs interact with each other. How exactly such an interaction takes place and how the overall properties are modulated in the axoneme will be important subjects of future studies.

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