

Functional Reconstitution of *Chlamydomonas* Outer Dynein Arms from α - β and γ Subunits: Requirement of a Third Factor

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Abstract. The outer dynein arm of *Chlamydomonas* flagella, when isolated under Mg^{2+} -free conditions, tends to dissociate into an 11 to 12S particle (12S dynein) containing the γ heavy chain and a 21S particle (called 18S dynein) containing the α and β heavy chains. We show here that functional outer arms can be reconstituted by the addition of 12S and 18S dyneins to the axonemes of the outer armless mutants *oda1-oda6*. A third factor that sediments at $\sim 7S$ is required for efficient reconstitution of the outer arms on the axonemes of *oda1* and *oda3*. However, this factor is not necessary for reconstitution on the axonemes of *oda2*, *oda4*, *oda5*, and *oda6*. SDS-PAGE analysis indi-

cates that the axonemes of the former two mutants lack a ~ 70 -kD polypeptide that is present in those of the other mutants as well as in the 7S fraction from the wild-type extract. Furthermore, electron micrographs of axonemal cross sections revealed that the latter four mutants, but not *oda1* or *oda3*, have small pointed structures on the outer doublets, at a position in cross section where outer arms normally occur. We suggest that the 7S factor constitutes the pointed structure on the outer doublets and facilitates attachment of the outer arm. The discovery of this structure raises a new question as to how the attachment site for the outer arm dynein is determined within the axoneme.

THE outer dynein arm of *Chlamydomonas* has three distinct heavy chains (α , β , and γ ; mol wt $> 500,000$) with ATPase activities and, when isolated, takes on a bouquetlike structure having three globular heads connected to a base (Johnson and Wall, 1983; Witman et al. 1983; Goodenough and Heuser, 1984). In the axoneme, these heads have been proposed to interact with the B-tubule in an ATP-sensitive manner, while the base is attached to the A-tubule in an ATP-insensitive manner (see Porter and Johnson, 1989). The *Chlamydomonas* outer arm tends to dissociate into "12S dynein" and "18S dynein" upon extraction with high salt solutions in the absence of Mg^{2+} . The 12S dynein is composed of the γ heavy chain and two light chains (Piperno and Luck, 1979), whereas the 18S dynein, having an actual sedimentation coefficient of ~ 21 S (Pfister and Witman, 1984), is composed of the α and β heavy chains, two intermediate chains (IC70 and IC78), and six light chains (Piperno and Luck, 1979; Pfister et al., 1982; King and Witman, 1988a, b). The two intermediate chains (IC)¹ and some of the light chains (LC) of 18S dynein make up a complex (IC/LC complex), which is tightly associated with the β heavy chain (King and Witman, 1990; Mitchell and Rosen-

baum, 1986). IC78 is thought to be located close to the A-tubule of the outer doublet, because the treatment of axonemes with a zero-length cross-linker links IC78 to α tubulin independently of the presence of ATP (King et al., 1991). It is likely that the IC/LC complex constitutes a site where the outer arm is attached to the A-tubule. However, the complex is apparently insufficient for the entire outer arm to bind to the doublet microtubule because 18S dynein contains the complex yet does not bind to axonemes in the absence of 12S dynein (Fay, R. B., and G. B. Witman, 1977. *J. Cell Biol.* 75: 286a; Takada et al., 1992).

We previously showed that the outer arm dynein extracted from the wild-type axoneme does not dissociate into 12S and 18S dyneins and remains as a three-headed dynein if sucrose density centrifugation of the crude dynein extract is performed in the presence of Mg^{2+} (Takada et al., 1992). Moreover, three-headed dynein can bind to the axoneme of an outer armless mutant, *oda1*, and restore a higher level of reactivated motility. In contrast, the 12S and 18S dyneins do not produce functional outer arms even when added together to the *oda1* axoneme. From these results, we have speculated that the dissociation of the outer arm into the 12S and 18S dyneins might be an irreversible process.

In this study, we show that the 12S and 18S dyneins can be recombined to form functional outer arms if mixed in the presence of a novel 7S factor that is present in the high-salt extract of the wild-type axoneme. This factor was missing from the axonemes of *oda1* and another outer armless mu-

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1. *Abbreviations used in this paper:* FFT, fast-Fourier-transform; IC, intermediate chain; LC, light chain; TAP, Tris-acetic acid-phosphate.

tant, *oda3*. However, it was present in those of the mutants *oda2*, *oda4*, *oda5*, and *oda6*, which also lack the outer arm. Electron micrographs of axonemal cross sections of the latter four *oda* mutants, but not of the former two mutants, frequently revealed small projections attached to the outer doublet at the site in the cross section where the outer arm normally occurs. These observations strongly suggest that the novel 7S factor forms a structure that facilitates attachment of outer arms to the A-tubule of outer doublets.

Materials and Methods

Strains

Chlamydomonas reinhardtii wild type (137c) and six strains of outer armless mutants (*oda1*, *oda2*, *oda3*, *oda4*, *oda5*, and *oda6*) (Kamiya, 1988) were used.

Culture of Cells

For preparation of cell models, cells were cultured in 200 ml of liquid Tris-acetic acid-phosphate (TAP) medium (Gorman and Levine, 1965) with aeration on a 14 h/10 h, light/dark cycle, to a final cell density of $2-4 \times 10^6$ /ml. For preparation of dynein samples, cells were cultured on 20 1.3% agar plates (600 cm²) containing TAP medium under continuous illumination at 23°C.

Isolation of 12S and 18S Dyneins

Cells grown on the TAP/agar plates for ~10 d were harvested and suspended in 4 liters of liquid TAP medium. The suspension was aerated under illumination for 10 h, during which period flagellar growth was complete. Flagellar axonemes were isolated essentially by the method of Witman (1986). The axonemes were extracted with a buffer solution containing 0.6 M KCl and the extract containing dynein was fractionated by sucrose density gradient centrifugation in TEDK solution (30 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM DTT, 25 mM KCl, 0.1 mM PMSF) as described previously (Takada et al., 1992). 20 fractions (250- μ l vol each) were collected and each was supplemented with MgSO₄ (final concentration: 5 mM) before use. The fractions containing 12S dynein and 18S dynein were those that sedimented at ~12 and ~21 S, respectively (Pfister and Witman, 1984).

Functional Assay of Dynein Preparations

Cell models were prepared using Nonidet P-40 (Kamiya and Witman, 1984). Activities of 12S and 18S dyneins to increase the reactivated motility of outer armless axonemes were assayed by measuring the beat frequency in *oda1* or *oda6* cell models in the following way. Cell models were mixed with the dynein sample at an appropriate volume ratio, left on ice for 60–90 min, and then reactivated in 0.5 mM ATP by mixing 2.5 μ l of the sample with 22.5 μ l of reactivation solution (20 μ l of 1.25-fold concentrated HMDEKP; 30 mM Hepes, 5 mM MgSO₄, 1 mM DTT, 0.5 mM EGTA, 50 mM K-acetate, 0.5% polyethylene glycol [mol wt = 20,000], pH 7.4; plus 2.5 μ l of 5 mM ATP). The volume ratio of the cell models and the dynein samples to be mixed was roughly adjusted so that the mixture of the 12S dynein, 18S dynein, and a 7S factor fraction (see Results) enhances the reactivated motility to a value close to the wild-type level. In most of the experiments, a cell model suspension was mixed with an equal volume of a dynein mixture, in which all or part of the above three fractions was contained in 1/3 amount each. The reactivated cell models were observed with a dark field microscope. The median flagellar beat frequency in a population of cell models was assessed by measuring the frequency distribution of the vibration of cell bodies in microscope images, using a photomultiplier and a fast-Fourier-transform (FFT) analyzer (Kamiya and Hasegawa, 1987). This method yields an estimate of the distribution of the flagellar beat frequency. As in our previous studies, the distribution patterns observed were roughly Gaussian, with estimated standard deviations of 10–20% (Takada et al., 1992).

Search for Factors That Promote Functional Reconstitution of Outer Dynein Arms

Each fraction from sucrose density gradient centrifugation or MonoQ

column chromatography was assayed for the activity to promote functional reconstitution of outer dynein arms. Part of each fraction was mixed with the 12S and 18S dynein fractions at equal volume ratios and added to the *oda1* cell models. The beat frequency of the reactivated cell model was measured after 60–90 min of mixing, as above.

Electron Microscopy

For observation of cross-sectional images of axonemes, cell models or isolated axonemes were fixed with 2% glutaraldehyde in the presence of 1% tannic acid, postfixed with 1% OsO₄, dehydrated through a series of ethanol solutions, embedded in Epon 812, and thin sectioned. The thin sections were double stained with uranyl acetate and lead citrate.

MonoQ Column Chromatography for Concentration of Active Components in 7S Factor

For partial purification of the protein components that have the activity to promote outer arm reconstitution in *oda1* axonemes, the 7S fraction from the wild-type axonemal extract and a high-salt extract from *oda6* axonemes were subjected to ion-exchange chromatography on a MonoQ column. With the wild-type 7S fraction, fractions 14–20 from three separate sucrose-density centrifuge tubes were pooled and applied to a MonoQ column. Proteins were eluted by using a step-elution protocol. With the *oda6* axonemal extract, crude dynein extract was first diluted 10-fold with HMDE solution (30 mM Hepes, 5 mM MgSO₄, 1 mM DTT, 1 mM EGTA, 0.1 mM PMSF, pH 7.4) to lower its ionic strength, applied to a MonoQ anion-exchange column, and eluted by a KCl gradient made in HMDE solution containing 0.1 mM PMSF. 0.5-ml fractions were collected. Each fraction was analyzed for protein composition by SDS-PAGE and for the activity to promote reconstitution of the outer arm function in *oda1* cell models.

SDS-PAGE

To examine dynein heavy chain bands by SDS-PAGE, 3–5% acrylamide gels with a 3–8 M urea gradient were used according to the method of Laemmli (1970) as modified by Jarvik and Rosenbaum (1980). For analysis of light or intermediate-sized chains, 5–20% acrylamide gradient gels were used.

Other Methods

Protein concentration was determined by the method of Bradford (1976).

Results

Effect of 12S and 18S Dyneins on the Reactivated Motility of *oda1* Cell Models

In a previous study we reported that the addition of the 12S outer arm dynein and 18S outer arm dynein fractionated from high-salt extract of wild-type axonemes did not enhance the reactivated motility of *oda1* cell models, even when added together. On the other hand, the addition of “three-headed” outer arms, prepared by a procedure that prevents dissociation of the three heavy chains, resulted in an increase in beat frequency from 28 to 53 Hz, a frequency close to that of wild type (Takada et al., 1992). To extend these findings, we first re-examined sucrose density fractions from the high-salt axonemal extract for their effects on the reactivated motility of the *oda1* cell model.

Fig. 1 shows the absorbance profile of the sucrose density gradient centrifugation of the extract, the SDS-PAGE pattern in the dynein heavy chain region for each fraction, and the beat frequency of reactivated *oda1* cell models to which an identical volume of each fraction had been added. The α and β heavy chains are mostly distributed in fractions 4–7 with a peak at fraction 6, while that of the γ chain is broadly distributed in fractions 7–14 (Fig. 1 B). The assay with *oda1* cell

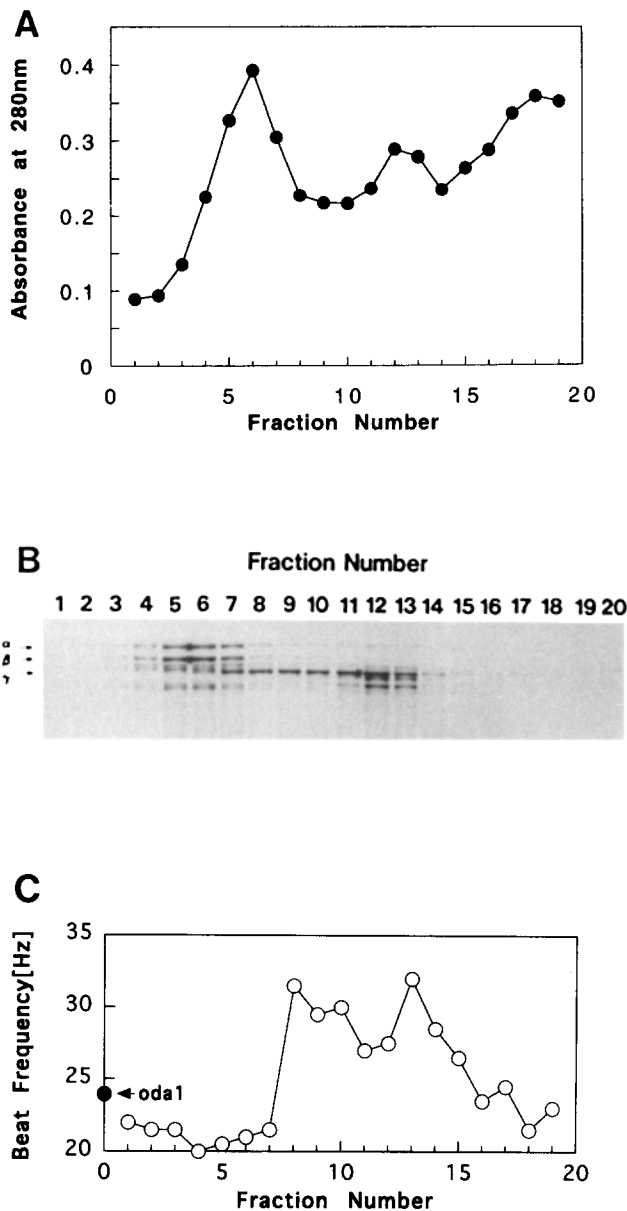


Figure 1. Fractionation of the high-salt extract from the wild-type axoneme by sucrose density gradient centrifugation and the effect of each fraction on the beat frequency of reactivated *oda1* cell models. (A) Absorbance profile at 280 nm of the sucrose gradient fractions. (B) SDS-PAGE pattern of each fraction showing the dynein heavy chain composition. (α , β , γ) Bands of outer arm dynein heavy chains. (C) Beat frequency of the reactivated *oda1* cell models to which an equal volume of each fraction had been added. The beat frequency shown was the median value in the power spectrum from the vibration of cell bodies, as analyzed with an FFT analyzer. The closed circle at the left end shows the frequency of control *oda1* cell models to which no fractions had been added.

models confirmed the previous observation in general, except that the fractions 8–15 containing the 12S outer arm dynein had an effect in increasing the reactivated motility by themselves; addition of these fractions increased the beat frequency by ~ 8 Hz (Fig. 1 C). In contrast to the 12S fraction, the 18S dynein fractions, fractions 4–7, did not increase the reactivated motility.

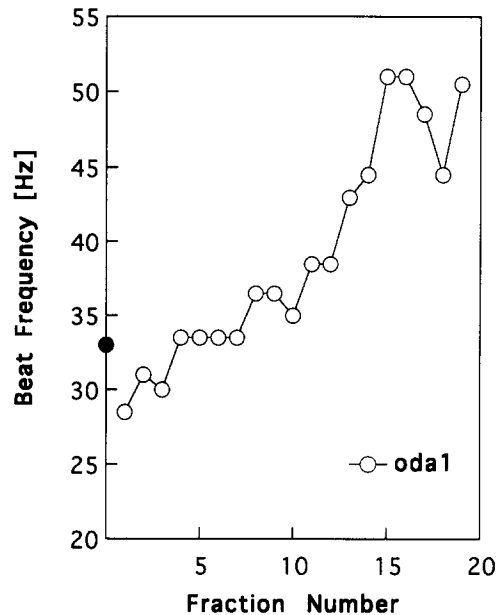


Figure 2. Effect of the simultaneous addition of 12S dynein, 18S dynein, and one of the sucrose density fractions to *oda1* cell models. A constant volume of each fraction was added to the *oda1* cell models in addition to the 12S dynein fraction and the 18S dynein fraction. After being kept for 1 h on ice, the cell models were reactivated in 0.5 mM ATP and their median flagellar beat frequencies were measured. Note that fractions 15 and 16 have a strong activity to enhance the beat frequency. The closed circle on the left end shows the beat frequency of cell models to which only the 12S dynein and the 18S dynein fractions had been added. The beat frequency of the control *oda1* cell models reactivated without added dyneins was 25 Hz (\circ *oda1*).

12S and 18S Dynein Greatly Increase the Beat Frequency When Combined with a 7S Fraction

We next examined to see if the simultaneous addition of the 12S dynein fraction and the 18S dynein fraction produces a functional outer arm and results in a still higher level of motility. The addition of a 1:1 (in volume) mixture of these two dynein fractions to the *oda1* cell models resulted in an increase in beat frequency by up to ~ 8 Hz (Fig. 2, filled circle). However, this increase was similar in magnitude to the one observed upon the addition of the 12S dynein fraction alone, and the final frequency attained was much lower than the wild-type value.

Suspecting that some additional factor is necessary for reconstitution of fully active outer arm dynein on the *oda1* axoneme, we examined whether some of the density gradient fractions has an activity to promote reconstitution. As shown in Fig. 2, we found that the beat frequency increased significantly when fractions 14–19 had been added in addition to 18S dynein (fraction 6) and 12S dynein (fraction 12). When fractions 15 and 16, sedimenting at ~ 7 S, had been added, the beat frequency became as high as 51 Hz, which is intermediate between the beat frequencies of the two flagella (*cis* and *trans*) in a single reactivated wild-type cell model (Kamiya and Witman, 1982; Kamiya and Hasegawa, 1987). The 7S fraction (fractions 15 and 16) itself did not increase the motility of the cell models noticeably (Fig. 1 C). We thus concluded that the 7S fraction contains a new fac-

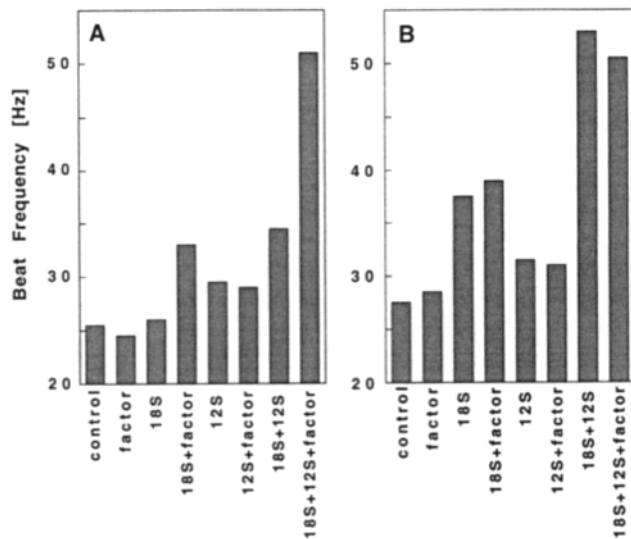


Figure 3. Effect of the 7S factor on the reconstitution of the outer dynein arm function on the cell models of *odal* (A) and *oda6* (B). The 12S dynein fraction, the 18S dynein fraction, or both, were added to the cell models with or without the 7S factor fraction. 60 to 90 min after the addition of these fractions, the cell models were reactivated and their median flagellar beat frequencies were measured. Beat frequencies of the cell models to which only buffer solution had been added were measured as controls.

tor(s) that promotes functional reconstitution of the outer dynein arm from 12S and 18S dyneins on the *odal* axoneme.

The addition of the mixture of the 7S fraction and the 18S dynein fraction to the *odal* cell models resulted in a moderate increase in the flagellar beat frequency, whereas the addition of the 7S factor or the 18S dynein fraction alone had no effect (Fig. 3 A). As stated above, 12S dynein fraction slightly enhances the motility of the *odal* axoneme by itself. The extent of the frequency increase caused by 12S dynein was unchanged by the presence of the 7S factor. Only when the two dynein fractions and the 7S fraction had been added simultaneously, did the beat frequency in *odal* cell models increase to the wild-type level.

The 7S Factor Promotes Association of Outer Arm Dynein to *odal* Axonemes

To see if the promotion of the functional restoration of outer arm by the 7S factor is due to a facilitation of binding of

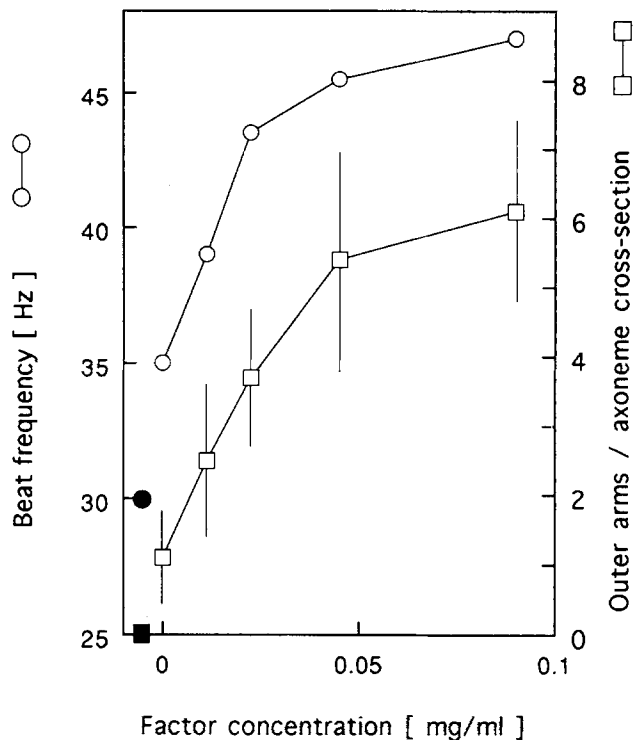


Figure 5. Simultaneous measurement of beat frequency and the number of outer arms on *odal* cell models mixed with dyneins and the factor. For samples, see Fig. 4 legend. Abscissa, the protein concentration of the 7S fraction when mixed with the cell models and dyneins. (○) Flagellar beat frequency in cell models. (●) Beat frequency in control *odal* cell models to which no dyneins had been added. (□ and bar) The number and standard deviation of the outer dynein arms occurring in axonemal cross sections. 10 to 13 electron micrographs were counted for each data point. (■) The number of outer arms in *odal* cell models to which no dyneins had been added; no outer dynein arms were found in 12 electron micrographs.

dynein to the outer doublets, we carried out electron microscopy on the *odal* cell models that were reactivated after having been mixed with varying amounts of the 7S factor together with constant amounts of 12S and 18S dyneins. As shown in Figs. 4 and 5, only one to two arms were found attached to the outer doublets when the two dynein fractions were added in the absence of the 7S factor, but up to an average of 6.1 outer arms appeared in the axonemal cross section

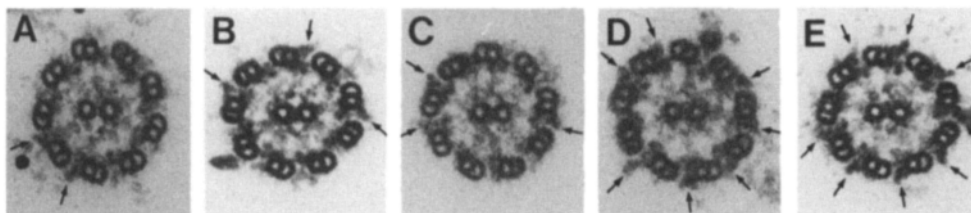


Figure 4. Electron micrographs of axonemes of *odal* cell models reactivated after having been mixed with 12S and 18S dyneins and the 7S factor. Cell models of *odal* were mixed with equal volumes of 12S dynein (0.36 mg/ml), 18S dynein (0.30

mg/ml) and a diluted sample of the 7S fraction, and kept in ice for 60 min. After the cell models were reactivated and the flagellar beat frequency was measured (Fig. 5), the cell models were centrifuged at 10,000 g for 15 min and the resulting pellet was fixed and processed for electron microscopy. (A) No factor had been added; (B) factor had been diluted eightfold; (C) fourfold; (D) twofold; (E) undiluted (0.36 mg/ml). Arrows indicate outer arms attached. Bar, 0.2 μ m.

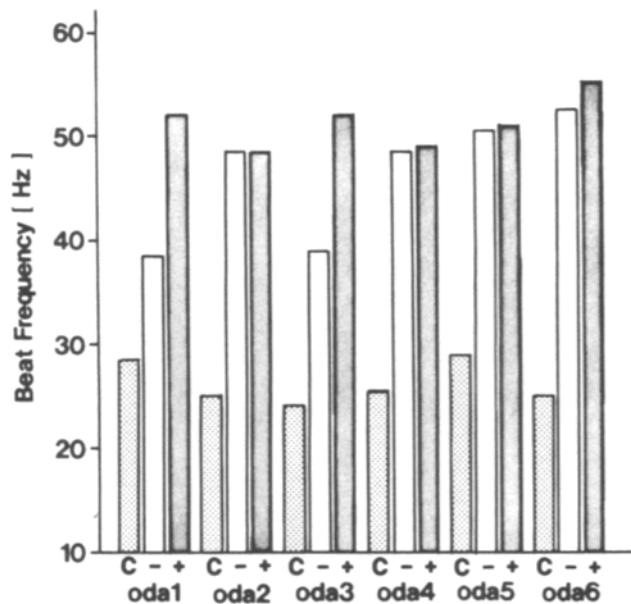


Figure 6. Requirement of the 7S factor for the restoration of functional outer arms in different *oda* mutants. Cell models of *oda1*, *oda2*, *oda3*, *oda4*, *oda5*, and *oda6* were mixed with equal volumes of the 12S dynein fraction, the 18S dynein fractions, and a buffer solution (-) or the 7S factor fraction (+). The beat frequencies before addition of the dyneins were shown by the bars labeled C. Note that the addition of the 7S factor causes a significant difference in flagellar beat frequency in *oda1* and *oda3*, but not in other *odas*.

when the 7S factor was added in addition to dyneins. The maximal number of outer arms occurring corresponds to 76% of that in the wild-type axoneme, which has eight outer arms per cross section (see Hoops and Witman, 1983). Importantly, the number of the outer arms per cross section depended on the concentration of the 7S factor, as did the beat frequency of reactivated cell models. The beat frequency and the number of the outer arms change in a roughly parallel manner (Fig. 5). Hence, we concluded that the effect of the 7S factor on the restoration of outer arm function in *oda1* axonemes is mostly through its promotion of dynein binding to the axoneme. We also observed that the beat frequency did not change noticeably when the mixture of the cell models, dyneins and the 7S factor had been left for varying time periods between 40 to 120 min before reactivation. This suggests that the factor functions in a nonenzymatic manner.

The 7S Factor Is Not Necessary for Outer Arm Reconstitution on *oda2*, *oda4*, *oda5*, and *oda6* Axonemes

The functional reconstitution of outer dynein arms on *oda1* axonemes thus requires a novel 7S factor in addition to 12S and 18S dyneins. We repeated the above experiment using another kind of outer arm-missing mutant, *oda6*, and was surprised to find that the 12S dynein fraction and the 18S dynein fraction can restore the wild-type motility even without the 7S factor; the addition of these two dynein fractions increased the frequency from 28 to 53 Hz (Fig. 3 B). Furthermore, the addition of 18S dynein alone increased the reactivated motility of the cell model to a level attained when

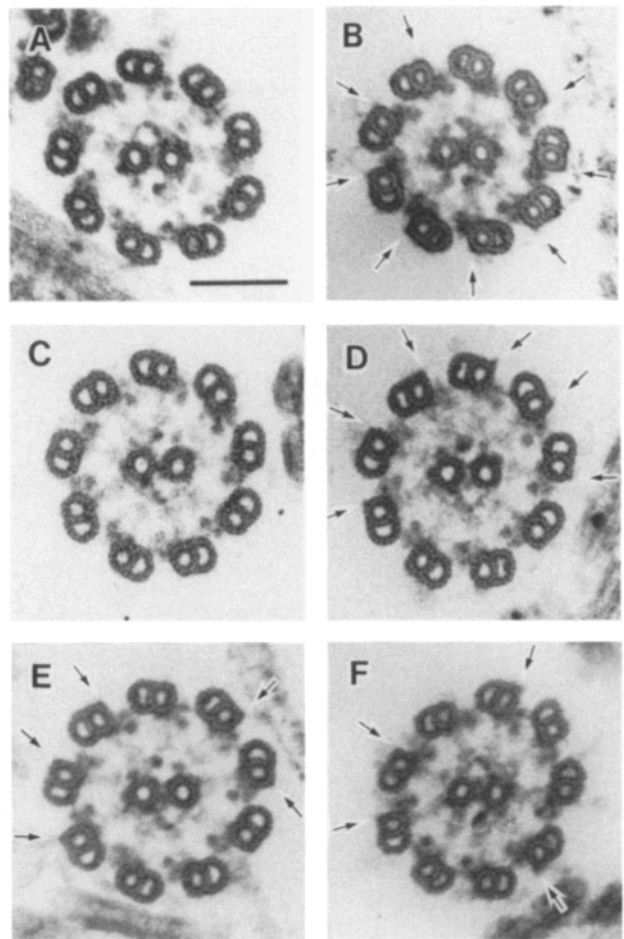


Figure 7. Electron micrographs of axonemal cross sections of different *oda* mutants. (A-F) *oda1-oda6*, respectively. Arrows indicate the small projections observed in *oda2*, *oda4*, *oda5*, and *oda6* mutants. Bar, 0.1 μ m.

18S dynein and the 7S factor were added together. Thus, the *oda6* axoneme apparently retains the 7S factor which is missing from the *oda1* axoneme.

We examined axonemes of four other *oda* strains and found that cell models of *oda3*, like those of *oda1*, require the factor for the flagellar beat frequency to increase to 50 Hz, but those of other *oda* strains do not (Fig. 6). The axonemes of the six *oda* strains tested can be thus classified into two types with respect to the requirement of the 7S factor. In a preliminary experiment, four other *oda* mutants, *oda7*, *oda8*, *oda9*, *oda10*, also did not require the 7S factor for restoration of outer arm function.

Structural Difference between Axonemes of Different *oda* Mutants

The above results indicate that the axonemes of different *oda* mutants somewhat differ, possibly in structure. In fact, we noticed a difference in published electron micrographs of *oda1-oda10* axonemes (Kamiya, 1988); the A-tubule of the outer doublets in *oda1* and *oda3* appeared smoothly round, whereas those of the outer doublets in other *oda* mutants ap-

Table 1. Occurrence of Small Projections on the Outer Doublets in Axonemes of Various *oda* Strains

Strains	Doublets with (+) or without (-) a projection			Doublets counted
	+	-	±	
	%	%	%*	
<i>oda1</i>	0.6	51.9	47.5	162 (18)‡
<i>oda2</i>	55.6	6.8	37.6	162 (18)
<i>oda3</i>	2.2	50.0	47.8	180 (20)
<i>oda4</i>	58.7	5.6	35.7	126 (14)
<i>oda5</i>	49.5	13.1	37.4	198 (22)
<i>oda6</i>	36.6	8.5	54.9	153 (17)

*Outer doublets on which the presence or absence of the projection is unclear.
‡Total numbers of the outer doublets and axonemes (in parentheses) measured.

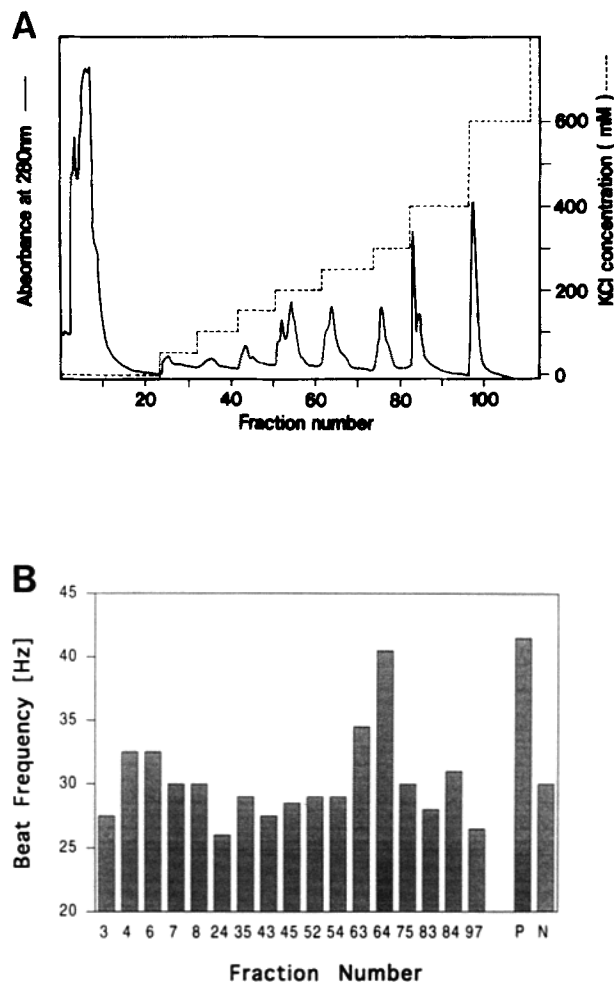


Figure 8. Ion-exchange chromatography of the 7S density gradient fraction. Fractions 14–20 from the sucrose density centrifugation were subjected to a MonoQ column and eluted by stepwise increase in KCl concentration. (A) Elution profile shown in relative absorbance at 280 nm. (B) Effect of each peak fraction on the restoration of outer arm function in *oda1* cell models. Each peak fraction was mixed with the *oda1* cell models together with the 12S and 18S dynein fractions and the beat frequency of reactivated cell models was measured. (P) Positive control in which *oda1* cell models had been mixed with the 12S and 18S dynein fractions and the 7S density gradient fraction. (N) Negative control in which the cell models were mixed with the 12S and 18S dynein fractions without the 7S fraction. Note that a strong activity is present in fractions 63–64, eluted at 250 mM KCl.

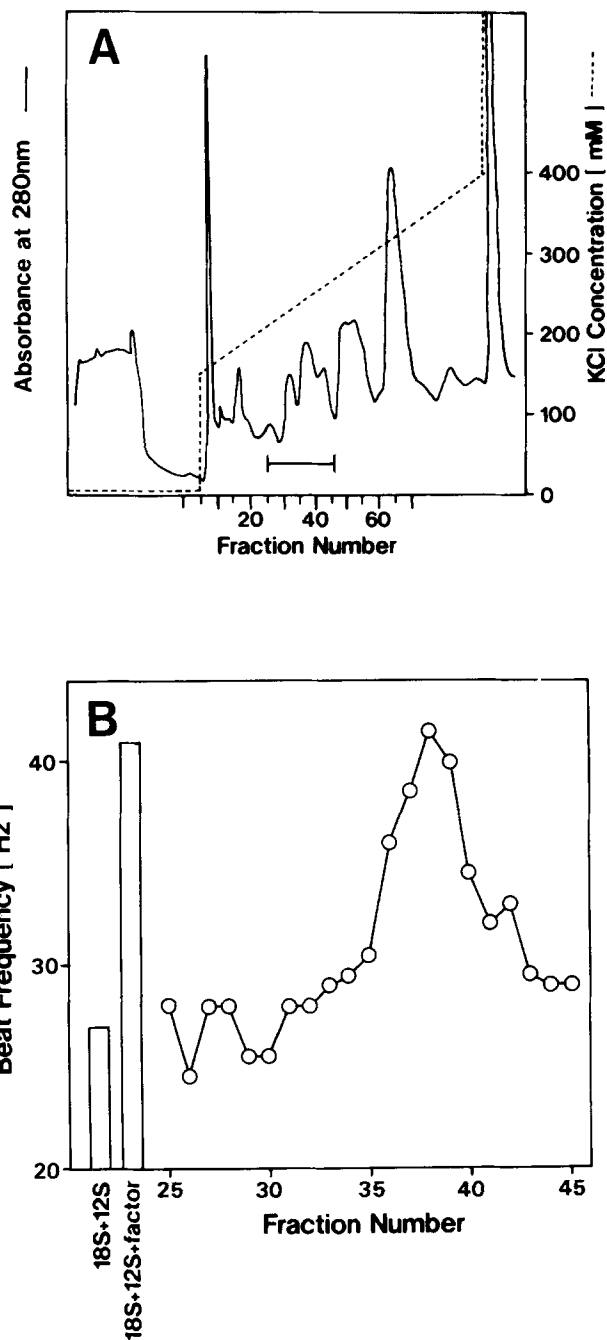


Figure 9. Ion-exchange chromatography of the extract from *oda6* axonemes. High-salt extract of *oda6* axonemes was subjected to a MonoQ ion-exchange column and eluted with a linear KCl gradient. (A) Absorbance profile at 280 nm. The 150–400 mM KCl gradient is shown by a dotted line. (B) The activity of fractions 25–45 (indicated by |—| in A) to promote outer arm restoration in vitro on *oda1* axonemes. Each fraction was mixed with the *oda1* cell models together with the 12S and 18S dynein fractions from the sucrose gradient. The motility of the cell models in the mixture were assayed. The bar labeled 18S + 12S is a negative control in which the 12S and 18S dynein fractions were added and the one labeled 18S + 12S + factor is a positive control in which the 12S dynein, the 18S dynein, and the 7S fraction from the sucrose gradient centrifugation were added to the cell models.

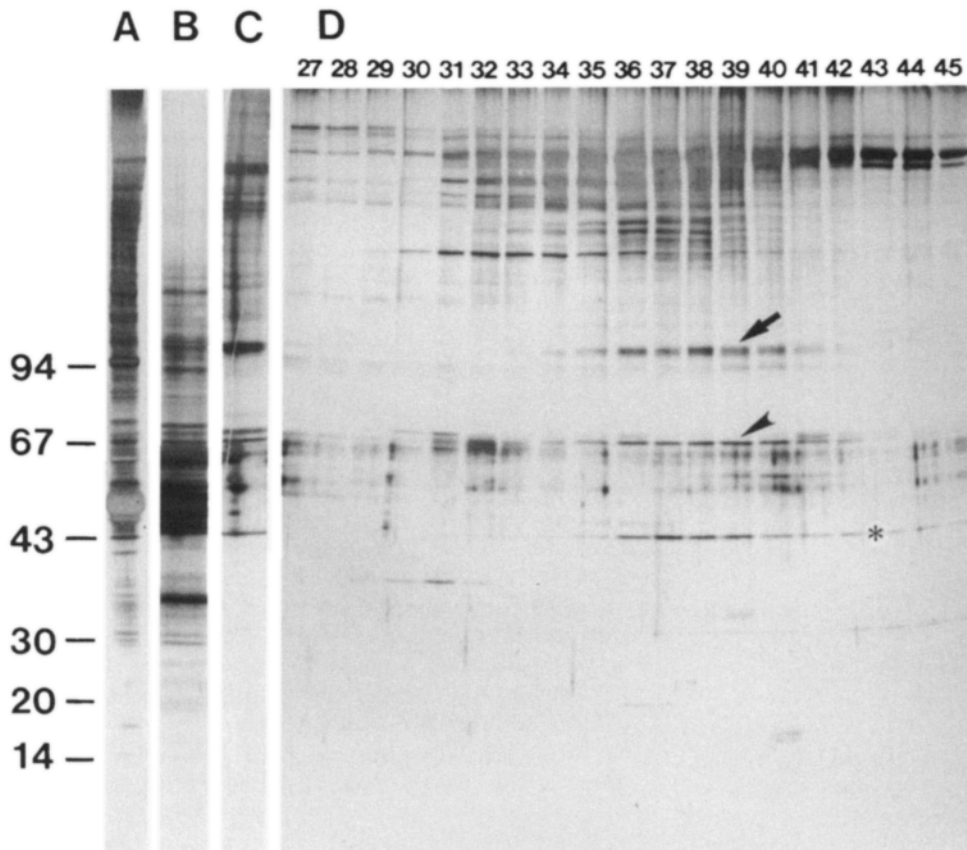


Figure 10. Polypeptide compositions in the concentrated fractions that have the activity to facilitate the outer arm restoration in vitro. (A) High-salt extract from the *oda6* axoneme. (B) The 7S fraction from the high-salt extract of wild-type axoneme. Fraction 16 in the sucrose density gradient centrifugation. (C) Fraction 64 in the MonoQ chromatography of the 7S fraction (Fig. 8). Samples loaded in B and C had roughly equivalent activities to promote outer-arm reconstitution in *odal* cell models. (D) Fractions from MonoQ chromatography of high-salt extract from *oda6* axonemes (Fig. 9). Note that a ~ 70 -kD band (arrowhead) is distributed around fraction 38 that has the strongest activity. This protein band is also present in A–C. Note also that another, slightly broad band at ~ 105 kD (arrow) appears to be distributed near fraction 38 and present also in A–C. A band at ~ 43 kD, marked by an asterisk, is that of actin, a compo-

nent of inner-arm dynein (see Piperno et al., 1990). This band is also present in the corresponding fraction from high-salt extract of *odal* axonemes, which has no activity to promote outer arm reconstitution. In sucrose density gradient centrifugation, the actin band is distributed around a 10S rather than 7S region.

peared to have a corner or a small projection at the site where outer dynein arms normally occur. To confirm this observation, we examined about 20 electron micrographs of axonemes for each of *oda1–oda6* and classified the outer doublet images into three categories: one that appears to have a corner or a small projection, one that appears to lack such a projection, and one that is not clear. Such an examination clearly indicated that outer doublets in *oda2*, *oda4*, *oda5*, and *oda6* axonemes frequently bear small projections at the sites where outer arms would be located in the wild-type axoneme, whereas such projections were almost never found in *odal* or *oda3* axonemes (Fig. 7; Table I). Thus, the requirement for the 7S factor for the restoration of outer arm function in *odal* and *oda3* is correlated with the absence of the small projection in their outer doublets.

A 70-kD Protein as an Active Component of the 7S Factor

The 7S factor used in the above experiments contained a number of proteins as judged by SDS-PAGE (see Fig. 10 B). To identify the protein(s) responsible for the effect of the 7S factor on outer arm reconstitution, the 7S fraction from the sucrose density gradient centrifugation of the wild-type extract was subfractionated by ion-exchange chromatography on a MonoQ column, using a step-elution protocol. The

fraction eluted at 250 mM KCl (fraction 64) had a strong activity to promote outer arm reconstitution (Fig. 8). This fraction still contained several proteins (Fig. 10 C).

Further purification of the factor from sucrose density fractions resulted in progressive loss of sample and made the motility assay unfeasible. We thus used another single-step method to purify the active factor(s) and compared its protein composition with that of the sucrose gradient centrifugation fraction. In this alternative procedure, high-salt extract from *oda6* axonemes was subjected to a MonoQ column. As in the MonoQ chromatography of wild-type extract, fractions eluted at ~ 250 mM KCl were found to promote the functional outer arm reconstitution on *odal* cell models (Fig. 9). The reconstitution-promoting activity in these fractions appeared closely correlated with the density of a ~ 70 -kD band in the SDS-PAGE pattern (Fig. 10 D). This polypeptide was also present in the active fraction partially purified from wild-type extract (Fig. 10, B and C). Furthermore, it was present in the high-salt extracts from the axonemes of *oda2*, *oda4*, *oda5*, and *oda6*, but was absent from those from axonemes of *odal* and *oda3* (Fig. 11). These results suggest that a ~ 70 -kD protein is at least one of the active protein components of the 7S factor.

In addition to the 70-kD protein, a polypeptide of ~ 105 kD was found in the active MonoQ fractions from wild type and *oda6* axonemes (Fig. 10), but it was absent in the corre-

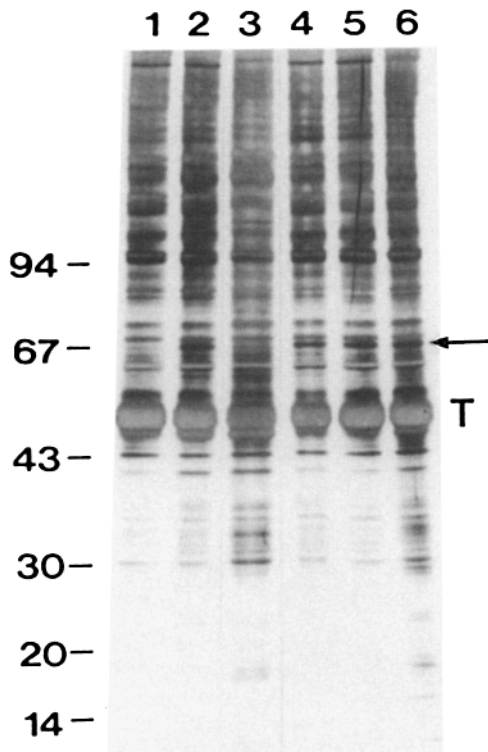


Figure 11. SDS-PAGE patterns of the high-salt extract from axonemes of different *oda* mutants. Supernates from the 0.6 M KCl extracts from *oda1-oda6* (corresponding to the lane numbers) axonemes were subjected to SDS-PAGE on a 5–20% gradient gel. Arrow indicates a ~70-kD band that is absent from *oda1* and *oda3* axonemes. (T) Tubulin.

sponding fractions from extracts of *oda1* or *oda3* axonemes (data not shown). Hence this protein also may be a component of the factor (see Discussion).

Discussion

Reconstruction of Functionally Active Outer Arms on Outer Arm-missing Axonemes

We have demonstrated that the function of outer dynein arm of *Chlamydomonas* flagella can be restored from its dissociated subparticles, i.e., 12S dynein containing the γ heavy chain and 18S dynein containing the α and β heavy chains. Using sea urchin sperm outer arm dynein, Tang et al. (1982) also attempted reconstituting outer arm dynein from its dissociated subparticles. Sea urchin outer arm dynein, with two heavy chains (α and β), dissociates upon dialysis against low ionic strength solutions into two subparticles with sedimentation coefficients of 10 and 15–17S. These authors showed that the dissociated subparticles reassociate to form a 21S particle when mixed under physiological ionic conditions. Its 21S sedimentation coefficient is the same as that of the intact outer arm. The re-formed 21S particle partially retains the ability to bind to axonemes, but does not restore the beat frequency of outer arm-depleted sperm axonemes. With *Chlamydomonas*, Fay and Witman (Fay, R. B., and G. B. Witman. 1977. *J. Cell Biol.* 75:286a) reported that neither

12S nor 18S dynein alone is capable of recombining with axonemes from which dyneins have been extracted with KCl solution, but that reassembly occurs when the two dyneins are mixed together. In this case also, functional restoration has not been demonstrated.

The present study is thus the first demonstration of functional reconstitution of dynein arm from dissociated particles. This method should provide means for studying the molecular mechanism of outer arm function, by perhaps allowing introduction of modified subunits. Combined use of this technique and appropriate mutants that lack particular outer arm heavy chains (Sakakibara et al., 1991, 1993) should enable detailed functional assay for each of the three heavy chains.

Factor Required for Reconstitution of Outer Arm Dynein on *oda1* Axonemes

An unexpected finding in the present study is that a novel factor sedimenting at ~7S is necessary for efficient reconstitution of the outer arm on *oda1* and *oda3* axonemes, but not for reconstitution on *oda2*, *oda4*, *oda5*, and *oda6* axonemes. Furthermore, electron microscopy of axonemal cross sections revealed a small pointed structure previously not recognized attached to the outer doublets. This structure is observed in the latter four mutants but not those of *oda1* and *oda3* axonemes. We suggest that the active factor partially purified from wild type or *oda6* axonemes is the solubilized form of this structure and probably constitutes an attachment site for the outer arm dynein.

The 7S factor and MonoQ fractions that have activities to promote outer arm reconstitution commonly contain a 70-kD protein, which is present in the axonemes of *oda2*, *oda4*, *oda5*, and *oda6*, but is absent from those of *oda1* and *oda3*. These findings strongly suggest that the 70-kD protein is the 7S factor itself or at least part of it. Luck and Piperno (1989), using two-dimensional electrophoresis, showed that a 73-kD polypeptide is missing in *oda1* and *oda3* but not in *oda2*, *oda4*, *oda5*, *oda6*, and wild-type axonemes. The 70-kD protein we found may be identical with their 73-kD protein because it is the only detectable difference in the 50–100-kD region of the SDS-PAGE patterns of different kinds of *oda* axonemes. In addition to the 70-kD polypeptide, a polypeptide of ~105 kD was found missing from the MonoQ fractions of *oda1* and *oda3* axonemal extracts eluted at 250 mM KCl, while it is present in the corresponding fractions from axonemal extracts of *oda6*. In SDS-PAGE patterns of the crude axoneme extracts, however, we have so far been unable to detect any difference in a molecular weight range near 105-kD, nor have Luck and Piperno (1989) described any difference in their analyses of *oda1-oda6* axoneme polypeptides using two-dimensional gel electrophoresis. Thus it is not certain whether axonemes of *oda1* and *oda3* lack this polypeptide also. However, we may expect that the 105-kD protein somehow interacts with the 70-kD protein, since it disappears from the corresponding MonoQ fraction when the latter protein is absent. Possibly, the small projection we found contains both proteins. At the present stage of purification, it is also possible that the 7S factor contains proteins other than these proteins.

Luck and Piperno (1989) showed that the 73-kD protein is a phosphoprotein with phosphorylation occurring on multiple levels. It would be interesting to examine the physiolog-

ical importance of the phosphorylation in the assembly and function of the outer arm.

A-tubule Binding Site of the Outer Dynein Arm

The experiment shown in Figs. 4 and 5 indicates that the 7S factor is necessary for outer dynein arms to bind to the axoneme. This finding is in apparent disagreement with our previous study (Takada et al., 1992) which showed that a three-headed dynein preparation can efficiently restore a wild-type level of motility when added to the *odal* cell models in the absence of added 7S factor (Takada et al., 1992). However, using two-dimensional electrophoresis, we have recently found that the three-headed dynein preparation contains a significant amount of the same 70-kD protein as that of the 7S factor (Takada, S., and T. Kato, unpublished result). Hence the efficient restoration of outer arm function with the three-headed dynein may be due to the presence of the 7S factor within the three-headed dynein. Whether the 7S factor is necessary for maintaining the integrity of the three-headed dynein remains to be established.

The finding that *odal* and *oda3* axonemes missing the 7S factor lack small projections on outer doublets suggests that the 7S factor provides a structure to which outer arm dynein binds. On the other hand, as stated in the Introduction, an intermediate chain, IC78, has been shown to be in direct contact with α -tubulin (King et al., 1991). Hence both of the 7S factor and IC78 may be involved in the binding of outer arm dynein to microtubules. We suggest that the 7S factor or the 70-kD protein is particularly important in determining the position where outer dynein arm occurs, because the projection in the mutants *oda2*, *oda4*, *oda5*, and *oda6* is apparently attached to the outer doublets at the right positions in cross section where outer arms occur (Fig. 7). Thus an important new question arises as to what structural features on the outer doublet specify the binding site for this factor. It would be of great interest to examine whether this factor binds to doublet and singlet microtubules in vitro, and if so, at what intervals it appears along the length of a microtubule.

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