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Binding and Adenosine Triphosphatase of Flagellar Proteins from Sea Urchin Sperm†

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ABSTRACT: The extracted ATPase from sea urchin sperm flagella, dynein, is found to rebind spontaneously to the outer fiber from 60 to 90% of the solubilized ATPase. The aggregate of 4S, 10S, and 13S components of crude dynein formed in 1 mM $CaCl_2$–30 mM KCl–20 mM Tris-HCl (pH 8.0) has the binding activity. The bound and unbound dyneins have different properties. K^+ ion activates the Ca-activated ATPase of dynein, but inhibits that of axoneme, and the high concentration of ATP inhibits the ATPase of axoneme, but does not inhibit that of dynein.

The glycerinated model of sperm showed the movement of flagella by the addition of ATP, suggesting that the movement of sperm tail was coupled to phosphohydrolysis of ATP (Hoffmann-Berling, 1955; Bishop and Hoffmann-Berling, 1959; Kinoshita, 1958, 1959; Brokaw, 1967). Flagella and cilia have usually the same arrangement of microtubles called the "9 + 2" system. Gibbons (1963) isolated the protein "dynein" having the ATPase activity from *Tetrahymena* cilia. This protein was identified as the arm projecting on the outer fiber of cilia and found to rebind specifically the outer fiber (Gibbons, 1963, 1965a). Some of its biochemical natures were examined (Gibbons, 1966). Cilia which rebound dynein showed the superprecipitation-like phenomenon by the addition of ATP (Gibbons, 1965b). It is certain that

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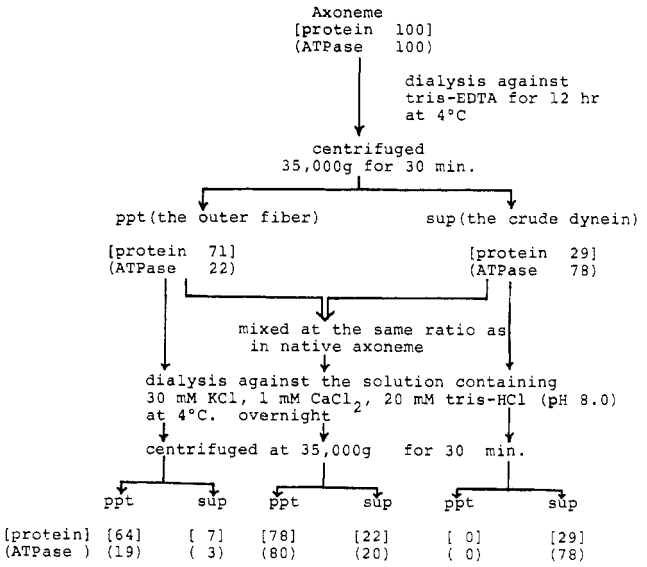

FIGURE 1: Summary of the procedure and the result of the extraction and the rebinding experiment of dynein. Total amounts of protein and ATPase are normalized to the original axoneme. The binding activity can be expressed as the ratio of the loss of the ATPase activity in the supernatant of the mixture to that of dynein (*i.e.*, (78% - 20%)/78% = 74% in this result). The ATPase activity was measured in 30 mM KCl–20 mM Tris HCl (pH 8.0)–1 mM $CaCl_2$–1 mM ATP at 20°.

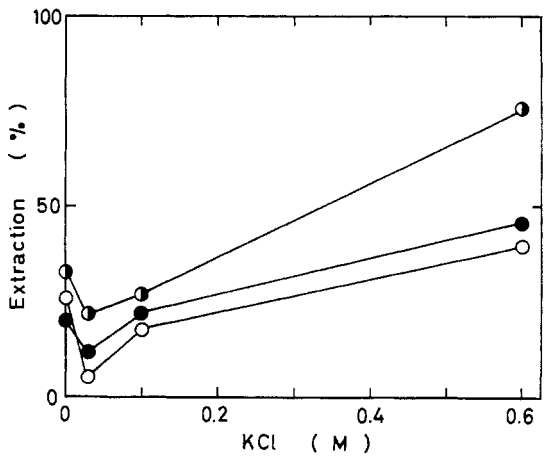

FIGURE 2: Extraction of the ATPase. Axonemes were incubated in designated concentrations of KCl in the absence and presence of 1 mM $CaCl_2$ or 1 mM $MgCl_2$ for 30 min at 4° and then centrifuged. ATPase activities of supernatants were measured in 0.6 M KCl–1 mM $MgCl_2$–1 mM ATP. The ATPase of the supernatant containing $CaCl_2$ was measured in 0.6 M KCl–1 mM $CaCl_2$–1 mM ATP and corrected by calculation to that in $MgCl_2$. ●: in the presence of 1 mM $CaCl_2$. ○: in the presence of 1 mM $MgCl_2$. ◑: in the absence of divalent cations.

the biochemical analysis of the interaction between dynein and the outer fiber will give the base for understanding the mechanism of beating of flagella and cilia.

The present study has been performed with this viewpoint using flagella from sea urchin sperm. The solubilized dynein can rebind to the outer fiber. When it binds, the ATPase activity of dynein is characteristically changed. There are remarkable differences between the free dynein and the bound dynein on the K^+ ion dependence of the Ca-activated ATPase and the inhibition by the substrate ATP. This fact seems to correspond to the effect of F-actin on the ATPase activity of myosin A from muscle and plasmodium.

Materials

Flagella. Sperm was collected from sea urchins, *Hemicentrotus pulcherrimus*, by injecting 0.5 M KCl. Ten volumes of filtrated cold sea water (pH 8.0) containing 0.1 mM EDTA were added, and the suspension was homogenized in a glass homogenizer to detach tails from heads. After removing heads by centrifugation at 2000 rpm for 15 min, tails (flagella) were sedimented by centrifugation at 8000 rpm for 20 min. Resuspension and centrifugation were repeated until heads were not found in the solution under a phase-contrast light microscope. The pellet of flagella was dissolved in 1 mM Tris–thioglycolate buffer (pH 8.0).

Axoneme. Axoneme was prepared from purified flagella according to Stephens and Linck (1969) with slight modifications. Flagella were suspended in a solution containing 1% Triton X-100–3 mM $MgCl_2$–30 mM Tris–thioglycolate buffer (pH 8.0) for 30 min at 0°. After washing by centrifugation at 10,000 rpm for 20 min, axonemes were separated from membrane fragments and solubilized substances. The structure of axoneme obtained was confirmed under an electron microscope with the negative staining method.

Crude Dynein and Outer Fiber. The procedure for separating crude dynein and the outer fiber was essentially the same as that of Gibbons (1965a). Axoneme was dialyzed against a solution of 0.1 mM EDTA and 1 mM Tris-HCl (pH 8.0) (Tris–EDTA solution) for several hours and then the dialysate was centrifuged at 35,000*g* for 30 min. A major part of the ATPase activity of the original axoneme was solubilized (crude dynein). The residue was washed three times by centrifugation at 12,000*g* for 30 min in a solution of 5 mM Tris-HCl (pH 8.0) and finally dissolved in 30 mM KCl–20 mM Tris-HCl (pH 8.0). Then only outer fibers were found in the solution under an electron microscope. All preparations were carried out at 0–4°.

Details of separation and rebinding of dynein and the outer fiber are described in Results.

Heavy Meromyosin. Heavy meromyosin was prepared from rabbit skeletal muscle according to the method of Szent-Györgyi (1953).

Methods

Protein concentrations were determined by the microbiuret method of Goa (1953) in the range of 0.2–1 mg/ml. The assay was standardized by bovine serum albumin.

The ATPase activity was measured by incubating the enzyme at 20° in 1 mM ATP under the solvent condition indicated in each case. After deproteinization by cold 7.2% perchloric acid, the amount of inorganic phosphate was determined by the method of Martin and Doty (1949).

Analytical ultracentrifugation was carried out by using a Spinco Model E.

ATP was obtained from Sigma Chemical Co., and other chemicals used were reagent grade (Katayama Chemical Co.).

Results

Extraction of Dynein. At first, according to the original method by Gibbons (1965a), axoneme dissolved in a solution containing 30 mM KCl–20 mM Tris-HCl (pH 8.0) was dialyzed against the Tris–EDTA solution. As shown in Figure 1, after

TABLE I: Ultracentrifugation of Crude Dynein.

Centrifugal Force (×g)	Time (min)	Remained ATPase in Supernatant (%)	Solvent Conditions
35,000	30	100	30 mM KCl–1 mM $CaCl_2$–20 mM Tris-HCl (pH 8.0)
59,000	40	100	30 mM KCl–1 mM $CaCl_2$–20 mM Tris-HCl (pH 8.0)
76,000	60	85	30 mM KCl–1 mM $CaCl_2$–20 mM Tris-HCl (pH 8.0)
105,000	60	50	30 mM KCl–1 mM $CaCl_2$–20 mM Tris-HCl (pH 8.0)
105,000	120	44	30 mM KCl–1 mM $CaCl_2$–20 mM Tris-HCl (pH 8.0)
105,000	120	93	1 mM Tris-HCl (pH 8.0)–0.1 mM EDTA

12 hr of the dialysis, about 80% of the total ATPase activity and 30% of the total protein were solubilized from the axoneme. The amount of the solubilized ATPase reached a maximum after 4.5 hr of dialysis.

The outer fiber separated from the extraction medium, however, retained about 20% of the original ATPase activity of the axoneme (Figure 1). This remaining ATPase was not easily removed from the fiber by various treatments, for example, by repeated washing, by the isoelectric precipitation at pH 5.6, or by the precipitation at 30% saturation of ammonium sulfate which contained 0.6 M KCl or 3 mM MgATP.

Next, solubilization of the ATPase protein was examined at various concentrations of KCl and divalent cations. In Figure 2, minimum solubility was found to be at 30 mM KCl. Divalent cations also inhibited its solubilization. Therefore, the condition, 30 mM KCl–1 mM $CaCl_2$–20 mM Tris-HCl (pH 8.0), was applied for the rebinding of dynein and the outer fiber as described later.

State of Dynein. No appreciable amount of crude dynein was sedimented in the presence or absence of low concentrations of salts by centrifugation at 35,000*g* for 30 min, which centrifugation was applied in the purification procedure and in the later experiment of the rebinding. However, centrifuging at higher speeds gave sediments. As summarized in Table I, in the Tris–EDTA solution, only 10% of the ATPase and protein were sedimented at 105,000*g* for 60 min. But in the presence of 30 mM KCl–1 mM $CaCl_2$–20 mM Tris-HCl (pH 8.0) at the same conditions of centrifugation, about 60% of the ATPase and 40% of the protein were sedimented. Under these conditions, dynein made aggregates which were spun down at 105,000*g*, but not at 35,000*g*.

The sedimentation pattern of the crude dynein gave three peaks of 4 S, 10 S, and 13 S in the Tris–EDTA solution. In 30 mM KCl–1 mM $CaCl_2$–20 mM Tris-HCl (pH 8.0), the same three peaks appeared. The area of these three peaks, however, particularly of 10 S and 13 S, was decreased, although a peak corresponding to the aggregate was not observed.

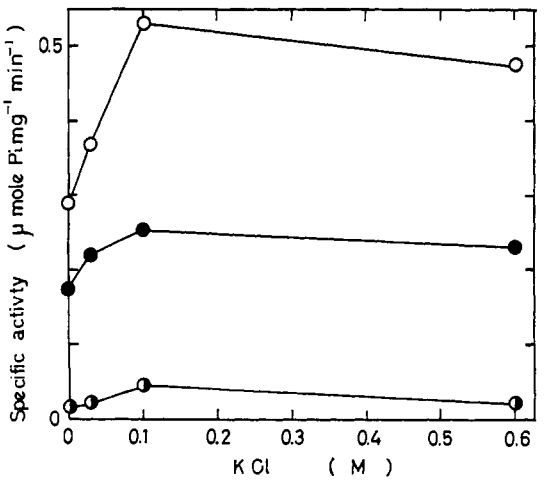

FIGURE 3: The ATPase of crude dynein. The ATPase activity of crude dynein was measured in various KCl concentrations containing 1 mM ATP and 20 mM Tris-HCl (pH 8.0) at 20°. ○: 1 mM $MgCl_2$. ●: 1 mM $CaCl_2$. ◑: no addition of divalent cation.

The effect of salts on crude dynein was reversible. After the removal of added salts by the dialysis against the Tris–EDTA solution, crude dynein was not sedimented at 105,000*g*. Once sedimented, the aggregates could not be solubilized in the Tris–EDTA solution, and the supernatant of the centrifugation at 105,000*g* in the presence of salts could not bind to the outer fiber.

ATPase Activities of Crude Dynein and Axoneme. The ATPase activity of the crude dynein solution was measured at various salt concentrations. As shown in Figure 3, in the absence of divalent cations a small maximum appeared at about 0.1 M KCl, and, in the presence of divalent cations, the activity increased with increasing KCl concentration. The ATPase activity was increased about 5-fold by the addition of 1 mM $CaCl_2$ and 20-fold by the addition of 1 mM $MgCl_2$, throughout the whole range of the KCl concentration examined. The maximum specific activity was about 0.5 μmole of P_i/mg per min in 0.1 M KCl–1 mM $MgCl_2$–1 mM ATP.

As shown in Figure 4, the dependence of the ATPase activity of axoneme on the salt concentration was the same as that of the crude dynein in the absence of divalent cation and in the presence of $MgCl_2$, although its specific activity was about 0.2 that of crude dynein. In the presence of $CaCl_2$, however, the ATPase activity of axoneme behaved in a different way from that of crude dynein. That is, in the absence of KCl, the ATPase activity of axoneme was higher in $CaCl_2$ than in $MgCl_2$ and this Ca-activated ATPase was decreased by the addition of KCl. From this difference in the KCl dependence of the Ca-activated ATPase between axoneme and crude dynein, it can be distinguished whether dynein is in the state of binding with the outer fiber or it is in the free state, as will be described in the study of rebinding.

The other remarkable property of the ATPase of axoneme was the inhibition at high concentrations of the substrate ATP, as shown in Figure 5. ADP and inorganic phosphate had no effect on this inhibition. In the case of crude dynein such a strong inhibition by ATP was not observed (Figure 6).

Binding of Dynein and Outer Fiber. An example of the rebinding experiment is shown in Figure 1. Crude dynein and the outer fiber in the Tris–EDTA solution were separated by the centrifugation at 35,000*g* for 30 min. Then they were mixed again at the same ratio as in the original axoneme, and

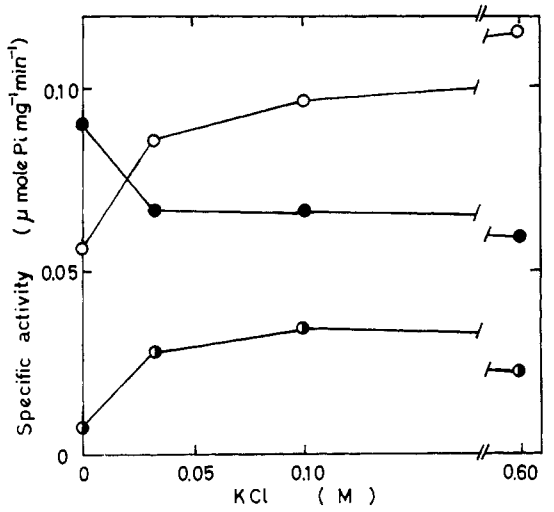

FIGURE 4: The ATPase of axoneme. The ATPase inhibited by K^+ ion was only found in the Ca-activated ATPase of axoneme. The reaction condition was the same as in Figure 3. ○: 1 mM $MgCl_2$. ●: 1 mM $CaCl_2$. ◑: no addition of divalent cation.

the mixture was dialyzed against the solution containing 1 mM $CaCl_2$–30 mM KCl–20 mM Tris-HCl (pH 8.0) at 4°. After overnight dialysis, the dialysate was centrifuged at 35,000*g* for 30 min. The ATPase activity and the amount of protein in the supernatant and the precipitate were measured. In the control experiments, solutions of the crude dynein and the outer fiber were separately dialyzed and centrifuged.

As shown in Figure 1, separated crude dynein was not sedimented by itself at the same salt concentration and the same

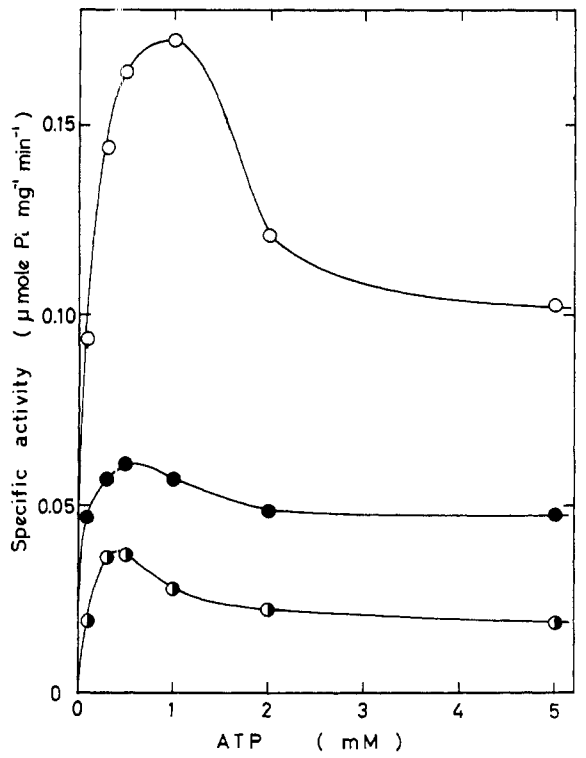

FIGURE 5: Substrate inhibition I. Axoneme. The reaction conditions were : 30 mM KCl–20 mM Tris-HCl (pH 8.0) and designated ATP concentrations at 20°. ○: 1 mM $MgCl_2$. ●: 1 mM $CaCl_2$. ◐: no addition of divalent cation.

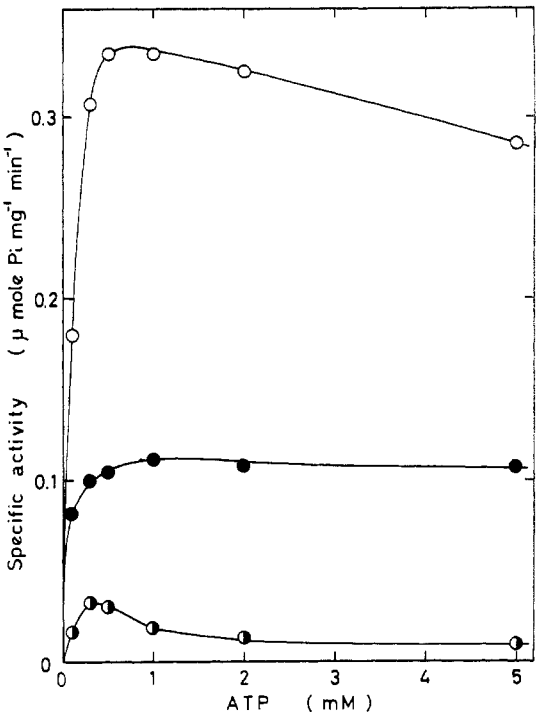

FIGURE 6: Substrate inhibition II. Crude dynein. The reaction conditions were the same as in Figure 5. ○: 1 mM $MgCl_2$. ●: 1 mM $CaCl_2$. ◑: no addition divalent cation.

conditions of centrifugation. Figure 1 also shows that only a small amount of ATPase was solubilized from the separated outer fiber. In the case of the mixture the ATPase of the supernatant was decreased from 78% to 20%, that is, about 78% − 20% = 58% ATPase was transferred to the precipitate. About 75% of solubilized ATPase was rebound to the outer fiber. It is unknown whether all the proteins rebound to the outer fiber had the ATPase or not.

The rebound dynein again recovered the same character of the ATPase activity as that of the native axoneme. As previously mentioned, the Ca-activated ATPase of the free dynein was activated by KCl but that of axoneme was inhibited by KCl. Figure 7 shows the KCl-dependent ATPase activities in the presence of 1 mM $CaCl_2$, of the original axoneme, the crude dynein, the outer fiber, and the axoneme reconstituted from crude dynein and the outer fiber. The ATPase of reconstituted axoneme returned to that of the native axoneme.

Association of the crude dynein and the outer fiber took place a few minutes after mixing and addition of salts. Further incubation did not increase the association.

For rebinding the presence of 1 mM $CaCl_2$ in addition to 30 mM KCl was more effective than 1 mM $MgCl_2$: for example, 50% binding occurred in 1 mM $CaCl_2$–30 mM KCl and only 35% binding in 1 mM $MgCl_2$–30 mM KCl.

As previously mentioned, crude dynein had 4 S, 10 S, and 13 S and made aggregates in the presence of salts. To confirm which component could bind, the original and the unbound fraction of crude dynein were analytically centrifuged (Figure 8). The unbound fraction of crude dynein was obtained by centrifugation at 35,000*g* for 30 min of the mixture of the outer fiber and crude dynein in the binding condition. Before analytical centrifugation, the supernatant (the unbound fraction) was dialyzed against the Tris–EDTA solution to bring it to the same condition as the original crude dynein. The area

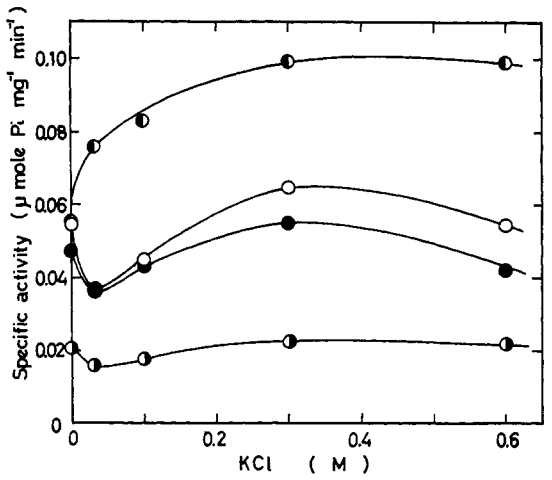

FIGURE 7: Recovery of the ATPase of the reconstituted axoneme. ATPase activities were measured in the designated KCl concentration–1 mM $CaCl_2$–20 mM Tris-HCl (pH 8.0)–1 mM ATP. The reconstituted axoneme was separated from the unbound dynein by centrifugation at 35,000*g* for 30 min. ○: original axoneme. ◐: extracted crude dynein. ◑: outer fiber fraction. ●: reconstituted axoneme (73% binding activity).

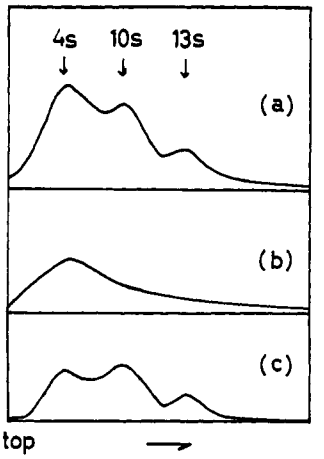

FIGURE 8: Schematic illustration of centrifugal pattern of bound dynein: (a) original dynein, (b) unbound dynein, (c) the pattern subtracted from a to b, which corresponds to that of bound dynein. Samples were centrifuged in the Tris–EDTA solution at 20°. Figures were traced from photographs taken after 30 min at 59,780 rpm and at a schlieren angel of 50°. Protein concentrations before centrifugation (a) 5.2 mg/ml, (b) 2.2 mg/ml, those of supernatant after centrifugation at 59,780 rpm for 54 min, (a) 4.3 mg/ml, (b) 1.7 mg/ml.

of the centrifugal pattern of the unbound dynein was about 50% of the original crude dynein. The difference of the centrifugal pattern between the original (Figure 8a) and the unbound fraction (Figure 8b) of crude dynein was shown in Figure 8c, which showed that three components of 4 S, 10 S, and 13 S contributed to the binding.

Crude dynein made aggregates in the presence of salt, which were sedimented at 105,000*g* for 60 min. The supernatant could no longer bind to the outer fiber. In addition, as shown in Figure 9 the unbound fraction of the crude dynein was not sedimented at 105,000*g* for 60 min in 1 mM $CaCl_2$–30 mM KCl–20 mM Tris-HCl (pH 8.0). It was concluded from the above results that the aggregates of 4 S, 10 S, and 13 S components of the crude dynein had the binding activity.

Heavy meromyosin from rabbit muscle did not bind to the outer fiber. When the outer fiber and heavy meromyosin were incubated in the binding condition above mentioned, the ATPase activity of the supernatant of centrifugation at 35,000*g* for 30 min was the same as that of the original heavy meromyosin. The pattern of the analytical centrifugation of the mixture of heavy meromyosin and the outer fiber was identical with that of heavy meromyosin itself.

Discussion

It has been proved that crude dynein can bind again to the outer fiber. The reversible binding of dynein was confirmed on the basis of the change of its ATPase activity.

Recently, Brokaw and Benedict (1971) found a difference in behavior of the ATPase between flagella and dynein from sea urchin sperm in the presence of urea or thiourea. Here, we found that the Ca-activated ATPase was different between axoneme and dynein in the physiological condition. The Ca-activated ATPase of axoneme and also reconstituted axoneme was inhibited by the addition of KCl, while that of dynein was activated by KCl. This difference between axoneme and dynein may be important in the flagellar movement.

In analytical centrifugation of dynein of flagella from sea urchin sperm, other workers found the 10–11S component (Mohri *et al.*, 1969) or the 11–14S component (Gibbons *et al.*, 1970) and the 4S component had no ATPase activity. In our case, crude dynein had 4S, 10S, and 13S peaks. In the presence of KCl and $CaCl_2$ small aggregates which were sedimentable by centrifugation at 105,000*g* for 1 hr were composed of these three components. Only this aggregate of crude dynein could bind to the outer fiber. The supernatant of the centrifugation at 105,000*g* could no longer bind. The unbound fraction of crude dynein was not sedimented at 105,000*g* for 1 hr in the binding condition. Then only the polymeric form of dynein seems to have the binding activity. Although the 4S component was reported to have no ATPase activity, in the present

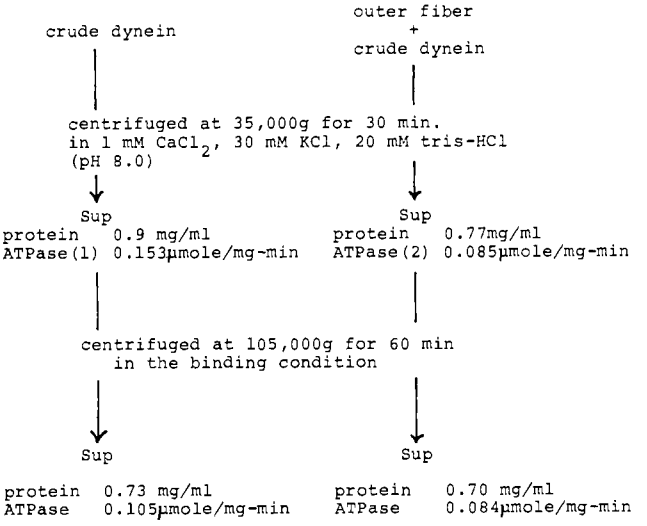

FIGURE 9: The effect of centrifugation on crude dynein and the unbound fraction of crude dynein. The binding activity was 44% (the ratio of (2):(1)). The results of the second centrifugation showed that the unbound fraction of the crude dynein (the supernatant from the first centrifugation of the mixture) did not form aggregates in the presence of salts, while in the supernatant of the crude dynein solution without the outer fiber, there appeared aggregates in the presence of salts.

experiment it was found that in the centrifugal pattern after the rebinding of dynein to the outer fiber, the area of this 4S component decreased. Therefore, presumably the light component (4 S) also participated in the binding and/or the polymerization of dynein. In the case of *Tetrahymena* dynein, only the polymeric form of 30 S had the binding activity and this component was separated from the 14S dynein in the stable state (Gibbons, 1963). However, this 30S component might incorporate the light component of 4 S.

On the electronmicroscopy of crude dynein, small particles of 100–150-Å diameter and also those aggregates were found in the presence of salts, but the rod-like polymers like the 30S form observed in the *Tetrahymena* dynein (Gibbons Rowe, 1965) could not be found.

Another significant finding was that the high concentration of substrate ATP inhibited markedly the ATPase activity of axoneme but only slightly that of the crude dynein. The activity of axoneme at high concentrations of ATP increased when an excess amount of $MgCl_2$ was added. This result suggests that the substrate of dynein is ATP and that of axoneme is the complex of ATP and Mg^{2+} ion. This property is very similar to that of muscle myosin A and actomyosin where the substrate of muscle myosin A is ATP and that of actomyosin is MgATP. Detailed studies on the enzymatic properties of dynein and axoneme will be reported elsewhere.

A paper which contains a similar result on the rebinding of dynein to the outer fiber using the sperm from the sea urchin *Pseudocentrotus depressus* appeared recently (Ogawa and Mohri, 1972).

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