Vol. 175, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1991 Pages 173-178

INNER ARM DYNEIN ATPASE FRACTION OF SEA URCHIN SPERM FLAGELLA CAUSES ACTIVE SLIDING OF AXONEMAL OUTER DOUBLET MICROTUBULE

Shigeo Wada, Makoto Okuno and Hideo Mohri

Department of Biology, College of Arts and Sciences, University of Tokyo, Komaba, Meguro-ku, Tokyo 153, Japan

Received January 18, 1991

In order to clarify the role of the inner arms of the axoneme in sperm flagellar movement, we prepared an ATPase fraction (12S) from the outer arm-depleted axonemes of sea urchin sperm flagella. When both arm-depleted axonemes were incubated with the 12S ATPase, they exhibited the sliding disintegration of outer doublet microtubules. Electron microscopy revealed that the ATPase rebound to the original inner arm sites of the axoneme. Therefore, it is quite likely that the 12S ATPase is one of the components of the inner arms. We referred to it as "inner arm dynein". © 1991 Academic Press, Inc.

Sliding of axonemal doublet microtubules is caused by the interaction among the outer doublet microtubules via their outer and inner arms, which are two rows of projections on doublet microtubules (1). The active components of the arms are known to be dyneins (2). In sea urchin sperm flagella, the outer arms are not always necessary for flagellar movement and microtubule sliding (3). Therefore, it has been assumed that the inner arms have important roles in causing the flagellar movement. The outer arm consists of 21S dynein ATPase (4). The biochemical and physiological properties of 21S dynein have been well characterized (5-8). The 21S dynein preparation causes the active sliding of the outer doublet microtubules (9) and also causes translocation of singlet microtubules (10,11).

Although Sale et al. (12) prepared an inner arm dynein from sea urchin sperm flagella, the physiological properties of the inner arms have not yet been well characterized. In this study we prepared an ATPase fraction from the inner arms that was capable of inducing the doublet microtubule sliding. Some biochemical properties were also characterized.

MATERIALS AND METHODS

The flagellar axonemes were prepared from the spermatozoa of the sea urchins *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus*.

The axonemes demembranated with Triton X-100 were mixed with a high salt extraction buffer (0.6 M KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM DTT, 20 mM Tris-HCl, pH 8.0) and incubated on ice for 30 min. The mixture was centrifuged at 13,000x*g*

0006-291X/91 $1.50
Copyright © 1991 by Academic Press, Inc.
All rights of reproduction in any form reserved.

for 10 min. The extraction was repeated again to remove the remaining outer arms completely. The pellet was washed and extracted with a low salt extraction buffer (1 mM Tris-HCl, 0.5 mM EDTA and 0.1 mM DTT, pH 8.0) for 30 min at 4°C. The mixture was centrifuged at 25,000x*g* for 20 min. The supernatant was concentrated up to five times by Aquacide III. Then it was applied onto a 5-20% sucrose density gradient (12m*l*) containing 1 mM Tris-HCl, 0.5 mM EDTA and 0.1 mM DTT, pH 8.0 and centrifuged for 20 h at 27,000 rpm in a Hitachi RPS-27-3 rotor. After centrifugation the gradient was fractionated and the peak fractions of ATPase activity were used as the ATPase component of the inner arm to analyze its properties.

The morphological recovery of the inner arm was examined by electron microscopy. The axonemes in which both arms were depleted from the outer doublet microtubules were prepared according to the method of Yano and Miki-Noumura (9). They were mixed with the ATPase prepared as described above in a rebinding buffer (0.15 M KCl, 4 mM $MgCl_2$, 0.5 mM EDTA, 10 mM Tris-HCl and 0.1 mM DTT, pH 8.0) and incubated for 60 min at 4°C. The mixture was centrifuged at 17,000x*g* and the pellet was fixed with glutaraldehyde, post-fixed with osmium tetroxide, embedded in Spurr, thin-sectioned and observed with a Hitachi HS9 electron microscope.

The motile activity was examined as follows. The semen was mixed with the demembranation solution (0.15 M KCl, 0.5 mM $MgSO_4$, 0.5 mM EDTA, 10 mM Tris-HCl, 0.04 % Triton X-100 and 0.5 mM DTT, pH 8.0) and kept for 30 s on ice with gentle stirring. The demembranated sperm suspension was mixed with the reactivation solution (0.15 M KCl, 2 mM $MgSO_4$, 2 mM EGTA, 20 mM Tris-HCl and 0.5 mM DTT, pH 8.0) without ATP. A drop of the suspension was applied to a perfusion chamber, and then the chamber was perfused from one end to the other with the low-salt extraction buffer to deplete both arms. After a 20 min extraction, the chamber was then perfused with rebinding solution to wash out the solubilized proteins followed by perfusion with the buffer containing inner arm ATPase (0.2 mg/ml) during a 20 min incubation. After washing with the rebinding buffer, the reactivation buffer containing 50μM ATP and 2μg/ml trypsin was applied. Movement of the outer doublet microtubules was examined by the dark-field microscopy.

Protein concentration was determined by absorbance at 280 nm, or by the method of Bradford (13). ATPase activity was assayed according to the method of Taussky and Shorr (14) with some modifications. SDS-PAGE was performed according to the method of Laemmli (15), with some modifications.

RESULTS AND DISCUSSION

Preparation of ATPase from the inner arm

The sperm flagellar axonemes were extracted twice with the high-salt extraction buffer in order to remove the outer arms completely. Electron microscopy revealed that almost all axonemes had no outer arms (<1%, data not shown). The outer arm-depleted axonemes were used for the preparation of the inner arm ATPase. When the low-salt extraction was carried out as described in Materials and Methods, the axonemes lost approximately 60% of their inner arms, while other structures such as nexin-links and radial spokes remained intact. Since the dominant ATPase activity of the axoneme is located in the arms (2), the major ATPase extracted with the low-salt buffer was assumed to come from the inner arms. A typical sedimentation pattern of the low-salt extract is shown in Fig. 1A. A symmetrical peak of ATPase activity (closed circles) was found, as indicated by the arrowhead in the figure. The estimated sedimentation coefficient of the peak was approximately 12S. Therefore we referred to it as 12S ATPase hereafter. The peak of ATPase activity coincided with the protein peak (open circles). The polypeptide composition of the fractions is shown in Fig. 1B. The 12S ATPase contained two major polypeptides, a high molecular weight (HMW) chain and an approximately 120kd polypeptide when 5.8% SDS-PAGE was carried out. Although

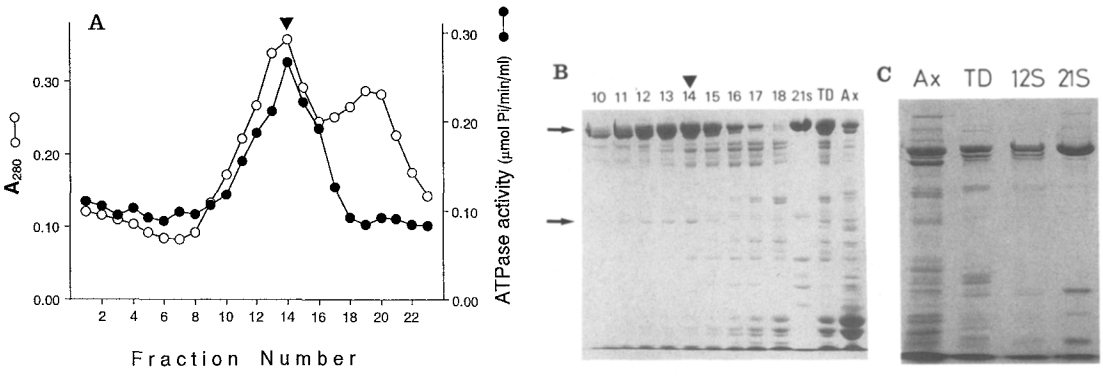

Figure 1. Preparation of inner arm ATPase from outer arm-depleted axonemes.
(A) 5-20% sucrose density gradient centrifugation fractionation of the low-salt extract. The open circles indicate the absorbance at 280nm and the closed circles indicate the ATPase activity.
(B) SDS-PAGE of fractions shown in A. SDS-PAGE was carried out with 5.8% acrylamide, 0.2% bis-acrylamide, containing 4 M urea.
Lanes 10-18: Fraction numbers corresponding to those in A are indicated at the top of the lanes. Lane 21s: 21S dynein of outer arm. Lane TD: Low-salt extract from outer arm-depleted axonemes. Lane Ax: Flagellar axonemes.
Arrowheads in A and B indicate the peak fraction of ATPase (Fraction No. 14), which has a sedimentation coefficient of about 12S. The peptides marked by arrows in B sedimented with the ATPase activity. These two peptides may be the components of the ATPase.
(C) High-resolution SDS-PAGE of fractions shown in B. SDS-PAGE was carried out with 3.9% acrylamide, 0.1% bis-acrylamide, containing 4 M urea. Lane Ax: Flagellar axonemes. Lane TD: Low salt extract from outer arm-depleted axonemes. Lane 21S: 21S dynein of outer arm. Lane 12S: Fraction No.14 in A.

there were also some other contaminant polypeptides, they sedimented more slowly than the 12S fraction. We further examined the composition of the HMW chain by high-resolution SDS-PAGE (Fig. 1C). The ATPase peak contained both A-band (similar to the A-band of the outer arm 21S dynein) and D-band observed in the HMW region of sea urchin sperm axonemes (16,17). However, the peptide mapping of the A-band polypeptides indicated that the A-band of 12S ATPase was different from that of 21S ATPase (data not shown).

Some properties of 12S ATPase

We investigated some properties of the 12S ATPase and compared them with those of the outer arm 21S dynein (Table 1). The specific activity of 12S ATPase was about 0.12 μmol/min/mg protein, which was smaller than that of 21S (1.0 μmol/min/mg protein). The ATPase activity of 12S ATPase gave the different Michaelis constant (K_m) and maximum velocity (V_{max}) from those of 21S dynein as shown in Table 1. Metavanadate ($NaVO_3$), which is known as a potent inhibitor of both 21S dynein ATPase activity and the reactivated flagellar movement, inhibited 12S ATPase strongly. Fifty percent inhibition of 12S ATPase was obtained at approximately 10^{-6} μM metavanadate, which is similar to that obtained with 21S dynein.

We tried to rebind 12S ATPase to both arm-depleted axonemes. When the low-salt extraction was carried out on the demembranated axonemes, only 0.3% of the outer arms and 14.1% of the inner arms were found to be remained by means of electron

Table 1. Some properties of 12S and 21S ATPase

	12S	21S
Peptide composition		
High molecular weight chains	A, D	Aα, Aβ
Intermediate chains	120kd	125kd, 90kd, 76kd
V_{max} (μmol Pi/min/mg)	0.13	1.1
K_m (μM)	12	46
Sliding activity of the microtubules	+	+

microscopy. After the incubation of the arm-depleted axonemes with 60 μg/ml of 12S ATPase, the axonemes regained the arm structures preferentially at the inner arm sites, because 38.3% of doublet microtubules displayed the inner arms, while only 0.4% of the doublet microtubules did the outer arms. On the other hand, the doublet microtubules regained only a few inner arms (7.0%) after incubation with 21S dynein (60 μg/ml). Therefore, 12S ATPase prefers to recombine with the inner arm sites of the arm-depleted axonemes rather than with the outer arm sites.

These results again indicate 12S ATPase to be the inner arm dynein of sea urchin sperm flagella and different from 21S outer arm dynein.

Microtubule sliding after recombination of 12S ATPase with both arm-depleted axoneme

Next we examined function of 12S ATPase in the axoneme by looking at the recovery of the activity to cause the sliding disintegration of outer doublet microtubules after incubation with the 12S ATPase. The outer doublet microtubules of the axoneme were depleted of both inner and outer arms with the low-salt extraction buffer. In this preparation, no sliding disintegration was observed when trypsin and ATP were added to the assay solution (Fig. 2A). On the other hand, when the arm-depleted axonemes were incubated with the 12S ATPase in the rebinding buffer, the axonemes were disintegrated by sliding of outer doublet microtubules a few minutes after the addition of ATP and trypsin (Fig. 2B). When they were incubated with the rebinding buffer alone, no sliding disintegration was observed (Fig. 2C). When the arm-depleted axonemes were incubated with 21S ATPase, the axonemes also disintegrated (data not shown), as Yano and Miki-Noumura reported previously (9). These results showed that 12S ATPase as well as 21S dynein gave rise the active sliding of the axonemal outer doublet microtubules.

The present experiment demonstrated that 12S ATPase rebound to the original inner arm site and caused active sliding of axonemal outer doublet microtubules. Therefore, the 12S ATPase must be a component of the inner arm of flagellar axoneme and play a part in the force-generating system in flagella. Recently, it was demonstrated that the

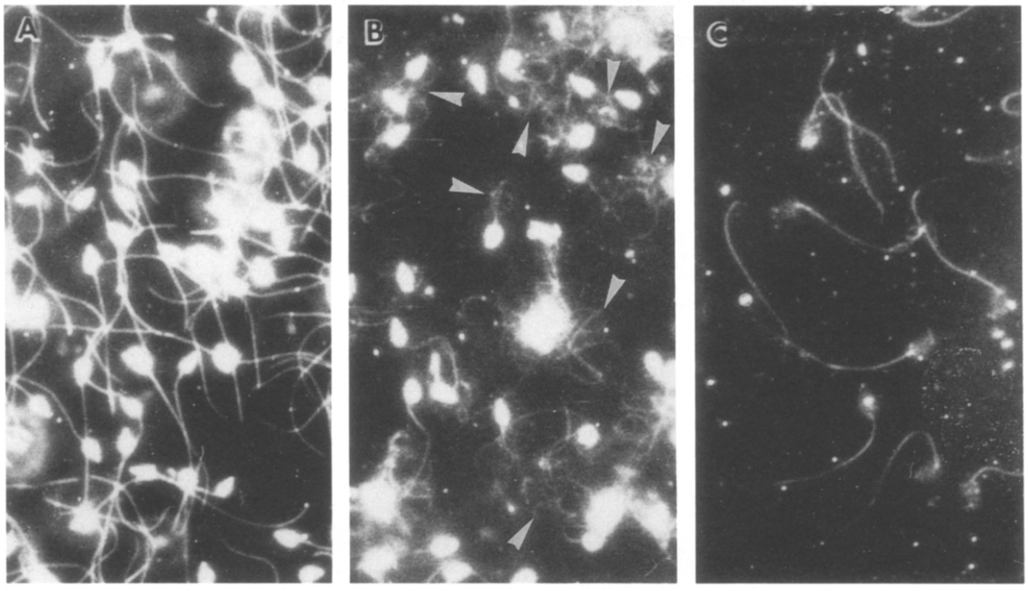

Figure 2. The active sliding of axonemal outer doublet microtubules recombined with the 12S ATPase fraction.
(A) The demembranated sperm flagella were extracted with the low-salt buffer for 20 min at 25°C and then 2 μg/ml trypsin and 0.05 mM ATP were added
(B) The arm-depleted sperm flagella (A) were incubated with 0.2 mg/ml of 12S ATPase fraction in the rebinding buffer for 20 min at 25°C. A few minutes after the addition of 2 μg/ml trypsin and 0.05 mM ATP, the axonemal outer doublet microtubules were disintegrated by sliding (arrowheads).
(C) The arm-depleted sperm flagella (A) were incubated with only the rebinding buffer for 20 min at 25°C. Even 20 min after the addition of 2μg/ml trypsin and 0.05 mM ATP, no sliding disintegration was observed.

inner arms consist of several heterogeneous molecules (18). The present 12S ATPase is undoubtedly one of the inner arm components.

ACKNOWLEDGMENT

We express our thanks to Mrs. T. Mohri for the electron microscopy.

REFERENCES

1. Summers, K. E. and Gibbons, I. R. (1971) Proc. Natl. Acad. Sci. USA, 68, 3092-3096.
2. Gibbons, I. R. and Rowe, A. J.(1065) Science, 149, 424-426.
3. Gibbons, B. H. and Gibbons, I. R. (1973) J. Cell Sci., 54, 337-357.
4. Johnson, K. A. (1985) Ann. Rev. Biophys. Biophys. Chem., 14, 161-188.
5. Gibbons, I. R. and Fronk, E. (1972) J. Cell Biol., 54, 365-381.
6. Lee-Eiford, A., Ow, R. A. and Gibbons, I. R. (1986) J. Biol. Chem., 261, 2337-2342.
7. Tang, W.-J. Y., Bell, C. W., Sale, W. S. and Gibbons, I. R. (1982) J. Biol. Chem., 257, 508-515.
8. Inaba, K. and Mohri, H. (1989) J. Biol. Chem., 264, 8384-8388.
9. Yano, Y. and Miki-Noumura, T. (1981) J. Cell Sci., 48, 223-239.
10. Paschal, B. M., King, S. M., Moss, A. G., Collins, C. A., Vallee, R. B. and Witman, G. B. (1987) Nature, 330, 672-674

11. Sale, W. S. and Fox, L. A. (1988) J. Cell Biol., 107, 1793-1797
12. Sale, W. S., Fox L.A. and Milgram, S.L. (1989) In Cell Movement (Warner, F. D. and McIntosh, J. R. Ed.), Vol. 1 pp. 89-108, Alan R. Liss, Inc., New York
13. Bradford, M. M. (1976) Anal. Biochem., 72, 248-254.
14. Taussky, H.H., and Shorr, E. (1953) J. Biol. Chem., 202, 675-685.
15. Laemmli, U. K. (1970) Nature, 227, 680-685.
16. Gibbons, I. R., Fronk, E., Gibbons B. H. and Ogawa K. (1976) Cell Motility (Goldman, R., Pollard, T., and Rosenbaum, J., Ed.), Book C. pp.915-935 Cold Spring Harbor Laboratory, Cold Spring Harbor
17. Ogawa, K. and Gibbons, I. R. (1976) J. Biol. Chem., 251, 5793-5801
18. Goodenough, U.W. and Heuser, J. E. (1985) J. Cell Biol., 100, 2008-2018