FUNCTIONAL RECOMBINATION OF DYNEIN 1 WITH DEMEMBRANATED SEA URCHIN SPERM PARTIALLY EXTRACTED WITH KCl

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SUMMARY: Demembranated sea urchin sperm were extracted with 0.5 M KCl as described earlier and reactivated in a solution containing 1 mM ATP. Their flagellar beat frequency was approximately 13 Hz, while that of standard reactivated sperm which had not been extracted with KCl was approximately 31 Hz at 23°C. Addition of soluble dynein 1 caused a gradual increase in the flagellar beat frequency to approximately 25 Hz after 10 min at room temperature. This restoration of frequency occurred in the absence or presence of ATP. Examination by electron microscopy showed that, whereas KCl-extracted sperm were lacking the majority of the outer arms on the doublet tubules, they had regained most of their outer arms following incubation with soluble dynein 1.

The preparation of demembranated sea urchin sperm by treatment with Triton X-100 has permitted us to manipulate the chemical environment of the axoneme in ways designed to elucidate the macromolecular mechanism of flagellar movement, for the motile apparatus remains intact and reactivatable with ATP (2,6,7). One valuable approach has involved the partial extraction of the dynein ATPase from the axonemal structure with 0.5 M KCl (3). This produces a decrease in the number of outer arms on the doublet tubules and a simultaneous decrease in the flagellar beat frequency. The results have demonstrated that the flagellar beat frequency is dependent upon the number of intact dynein arms present on the axonemes, and constitute strong evidence for a functional role of the arms in the mechanism of motility.

Salt extraction has long been a standard procedure for the preparation of soluble dynein ATPase (3,8,11). Recently it has been shown that dynein in sea urchin sperm flagella exists in two isoenzymic forms named dynein 1 and dynein 2 (9,12). Dynein 1 constitutes the largest proportion of the axonemal ATPase activity and can be obtained in large quantity from axonemes of sperm from the sea urchin Tripneustes gratilla.

In the preliminary experiments reported here, we have studied the recombination of soluble dynein 1 with KCl-extracted sperm and have found that their frequency is restored to 80% of that of standard reactivated sperm, with accompanying reappearance of the outer arms on the doublet tubules.

METHODS: Soluble dynein 1 was prepared from sperm of the sea urchin Tripneustes gratilla. Axonemes were isolated by the procedure of Gibbons, I. R. and Fronk (8) with the modification that 1 mM $CaCl_2$ was added to all solutions and they were buffered with 5 mM imidazole, pH 7.0, instead of Tris. An extra wash of the axonemal pellet was also added to insure complete removal of Triton X-100. Soluble dynein 1 was then extracted from the axonemes by treating them for 5 min at 4°C with 0.5 M NaCl, 4 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM dithiothreitol, 3 mM mercaptoethanol, and 5 mM imidazole buffer, pH 7.0.

KCl-extracted, reactivated sperm were prepared by adding 25 µl of a suspension of sperm from the sea urchin Colobocentrotus atratus (approx. 3 mg protein/ml) to 0.3 ml demembranating solution containing 0.5 M KCl, 2 mM $MgSO_4$, 0.5 mM EDTA, 1 mM dithiothreitol, 0.04% Triton X-100 (wt./vol.), 10 mM Tris-HCl buffer, pH 8.1 at room temperature (23°C). The extraction was terminated after 50-60 sec by diluting 5-10 µl of the sperm suspension into a relatively large volume (2.5 ml) of reactivating solution containing 0.15 M KCl, 2 mM $MgSO_4$, 0.5 mM EDTA, 1 mM dithiothreitol, 10 mM Tris-HCl buffer, pH 8.1 and an aliquot of soluble dynein 1 prepared as described above. This mixture of KCl-extracted sperm in reactivating solution containing dynein 1 was incubated at room temperature as described in Results for individual experiments, ATP was added to a concentration of 1 mM and the sperm were observed visually by dark-field light microscopy. The beat frequencies of sperm swimming freely in Formvar-coated dishes were measured at 23°C as described earlier (2).

Samples of sperm to be examined by electron microscopy were collected by centrifuging the suspension of sperm in reactivating solution at 16,000 g for 10 min. The resultant pellets were fixed for 1-1/2 hours in 2% glutaraldehyde in phosphate buffer at pH 7.6 and treated further as described earlier (2).

RESULTS

As reported earlier (3), extraction of demembranated sea urchin sperm with 0.5 M KCl lowers their beat frequency upon reactivation in 1 mM ATP from approximately 31 Hz to 13 Hz. Moreover, this reduction is uniform and reproducible, and the form of the flagellar bending waves is similar to that of standard reactivated sperm.

Experiments to study the possible recombination of soluble dynein 1 with KCl-extracted sperm were performed with dynein from sperm of the sea urchin Tripneustes gratilla, because it was routinely being prepared in large quantity in our laboratory by Mr. Earl Fronk. The most satisfactory preparations of KCl-extracted sperm, however, were obtained using sperm of the sea urchin Colobocentrotus atratus. The beat frequency of demembranated, KCl-extracted

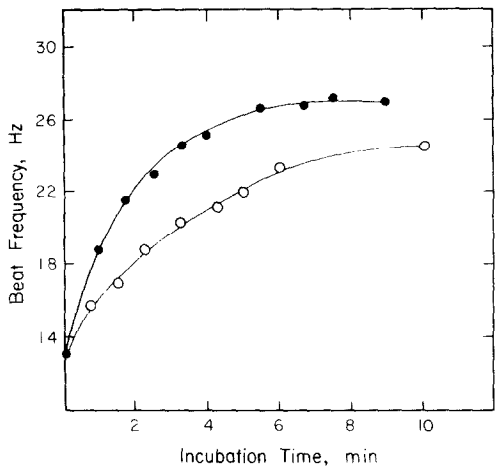

Fig. 1 Increase in the beat frequency of KCl-extracted sperm with the time of incubation with 10 µl (o) or 25 µl (•) soluble dynein 1 in the presence of 1 mM ATP. Values for each curve were obtained by following a single sperm. Temperature, 23°C.

sperm from Colobocentrotus can be restored by incubating them with soluble dynein 1 from Tripneustes in the presence of ATP as shown in Fig. 1. The rate of the increase is higher with greater amounts of dynein 1, but it also varies considerably with individual sperm. The incubation may also be done in the absence of ATP, and the beat frequency attained upon subsequent addition of 1 mM ATP is 3-4 Hz higher than after incubation in the presence of ATP. The maximum frequency obtained with a saturating amount of dynein 1 incubated in the absence of ATP was typically around 25 Hz.

The structural recombination of soluble dynein 1 with KCl-extracted axonemes was studied with the electron microscope. Figure 2 shows that whereas the KCl-extracted sperm used as starting material for the recombination experiments were lacking the majority of their outer arms on the doublet tubules, they had regained most of their outer arms following incubation with soluble dynein. A blind count of cross sections in a typical preparation photographed in the electron microscope showed 7% of the outer arms present before and 77% after incubation with dynein 1. A count of the outer arms present in a preparation of standard reactivated sperm gave a value of 95%.

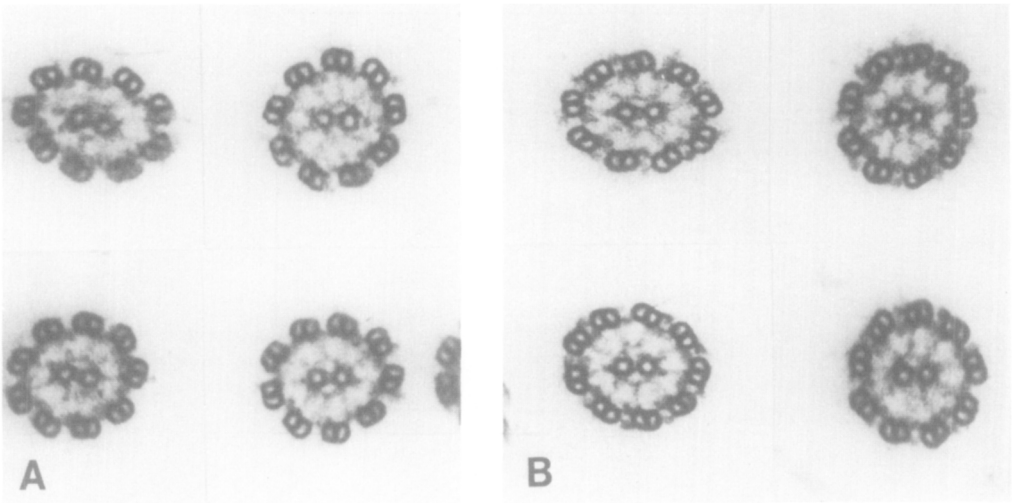

Fig. 2 Electron micrographs showing cross sections of axonemes which have been extracted with 0.5 M KCl. A, a preparation of sperm to which no soluble dynein 1 was added; a sample of these sperm added to reactivating sperm containing 1 mM ATP beat at 15 Hz. B, sperm from the same preparation following incubation with 250 µl soluble dynein 1 for 6 min. The beat frequency of a sample of these sperm was 25 Hz.

We have also incubated standard reactivated sperm (2) with 100 µl of soluble dynein 1 for 10 min in a Petri dish as described for KCl-extracted sperm in Methods. There appeared to be a slight increase in beat frequency (average 1 Hz in 4 preparations), but this was barely above the experimental error.

The preparations of soluble dynein 1 used for this work have been subjected to SDS gel electrophoresis and shown to contain principally the high molecular weight polypeptide known as the A band of dynein (9). In addition there are small amounts of two higher molecular weight proteins plus three lower molecular weight proteins, all of which appear to be tightly associated with the A-band protein, as well as some tubulin. The significance of these minor protein components is not yet known.

Soluble dynein 1 prepared in the manner described here has a low specific ATPase activity and has been called latent activity dynein 1 (9). The ATPase

activity of this form can be increased 5-10 fold by a variety of activating treatments. Preliminary experiments suggest that the activated form of dynein 1 has lost its ability to restore the beat frequency of KCl-extracted sperm, and that the latent activity form is the more physiological one.

DISCUSSION

This study demonstrates clearly that soluble dynein 1 can recombine with KCl-extracted sea urchin sperm, and that this recombination results in the reconstitution of functionally active dynein arms on the outer doublet tubules. The beat frequency of the KCl-extracted sperm reactivated in 1 mM ATP rises from approximately 13 to 25 Hz upon incubation with soluble dynein 1.

It is not clear why the restored beat frequency is somewhat less than the beat frequency of standard reactivated sperm. The restored value was not increased significantly by using larger quantities of dynein 1 or by allowing a longer time for recombination. The electron microscope observations indicate that some of the sites are simply missing a reconstituted arm, suggesting that a certain proportion of the dynein arm sites on the A tubules do not bind soluble dynein 1 for some reason, perhaps because they are denatured or blocked in some way. The results also suggest that some reconstituted arms may be non-functional, since counts of the total number of arms on recombined axonemes (85-90%) usually are slightly higher than the beat frequency (80%) when expressed as a percentage of the corresponding figures for standard reactivated sperm. The presence of ATP in the incubation medium interferes slightly with the recombination, but it is not known what the effect is. Perhaps active sliding of the tubules tends to prevent the binding of dynein 1. Fronk and I. R. Gibbons (manuscript in preparation) have obtained essentially complete structural reconstitution of dynein arms as seen in electron micrographs by recombining soluble dynein 1 with NaCl-extracted Tripneustes axonemes, but in this case it is not possible to correlate the data with the beat frequency, for these axonemes are non-motile.

Several other investigators have studied the recombination of dynein ATPase

with the outer fibers of both cilia and flagella. I. R. Gibbons recombined dynein ATPase with Tetrahymena axonemes and demonstrated that the rebinding was associated with the reappearance of arms on the outer doublet tubules and with recovery of the sensitivity of the turbidity to ATP (4,5). Shimizu has confirmed these results and reported that ATP had an inhibitory effect on the recombination (14). Binding of dynein ATPase to outer fibers of sea urchin sperm has also been reported (10,13). A detailed study by Blum and Hayes describes the effect of N-ethylmaleimide and heat treatment on the binding of dynein to Tetrahymena axonemes (1), and the results are in agreement with our finding that only the latent activity form of dynein 1 and not the activated form can be recombined with axonemes. The work reported here is the first to show that recombination of dynein ATPase with outer doublet tubules can restore functional activity to the axoneme.

The results of this work not only provide further evidence that the arms are composed of dynein 1, but also that they are directly involved in the mechanism of motility. Moreover, the recombination system provides an important method for assaying the functional activity of dynein 1 preparations.

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References

1. Blum, J. J. and Hayes, A. (1974) Biochemistry 13, 4290-4298.
2. Gibbons, B. H. and Gibbons, I. R. (1972) J. Cell Biol. 54, 75-97.
3. Gibbons, B. H. and Gibbons, I. R. (1973) J. Cell Sci. 13, 337-357.
4. Gibbons, I. R. (1965) Arch. Biol. (Liège) 76, 317-352.
5. Gibbons, I. R. (1965) J. Cell Biol. 26, 706-712.
6. Gibbons, I. R. (1974) In The Functional Anatomy of the Spermatozoon (B. A. Afzelius, editor). Pergamon Press, Oxford. pp. 127-140.
7. Gibbons, I. R. (1975) In Molecules and Cell Movement (S. Inoué and R. E. Stephens, editors). Raven Press, New York. pp. 207-231.
8. Gibbons, I. R. and Fronk, E. (1972) J. Cell Biol. 54, 365-381.
9. Gibbons, I. R., Fronk, E., Gibbons, B. H. and Ogawa, K. (1976) In Cell Motility (R. Goldman, T. Pollard and J. Rosenbaum, editors). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. (In press).
10. Hayashi, M. and Higashi-Fujime, S. (1972) Biochemistry 11, 2977-2982.
11. Kincaid, H. L., Gibbons, B. H. and Gibbons, I. R. (1973) J. Supramol. Struct. 1, 461-470.
12. Ogawa, K. and Gibbons, I. R. (1976) J. Biol. Chem. (In press).
13. Ogawa, K. and Mohri, H. (1972) Biochim. Biophys. Acta 256, 142-155.
14. Shimizu, T. (1975) J. Biochem. (Tokyo) 78, 41-49.