Bending Motion of *Chlamydomonas* Axonemes after Extrusion of Central-Pair Microtubules

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Abstract. Relatively little is known about the functions of central-pair microtubules (Tamm, S. L., and G. A. Horridge, 1970, *Proc. Roy. Soc. Lond. B*, 175: 219–233; Omoto, C. K., and C. Kung, 1979, *Nature (Lond.)*. 279:532–534) and radial spokes (Warner, F. D., and P. Satir, 1974, *J. Cell Biol.*, 63:35–63), although a sliding microtubule mechanism has been established for the flagellar movement (Summers, K. E., and I. R. Gibbons, 1971, *Proc. Natl. Acad. Sci. USA.*, 68:3092–3096).

In the present report, an attempt was made to determine the functions of central-pair microtubules in flagellar motility. Central-pair microtubules were found to extrude from the tips of elastase-digested axonemes of demembranated *Chlamydomonas* flagella after the addition of ATP. The length of the extruded central-pair microtubules was ~70–100% that of the axoneme. After extrusion, axonemes continued to swim slowly backwards in the reactivation medium, with a trailing central pair attached like a tail to the flagellar tip. During bending movement of the axonemes, partially extruded central pairs rotated counterclockwise about the axoneme axis, as viewed from the distal end (Kamiya, R., 1982, *Cell Motil.* [Suppl.]:169–173). Axonemes swam backwards with a symmetric waveform and a beat frequency of ~10 Hz in the reactivation medium containing 10^{-9}–10^{-4} M Ca ions. Even at a lower Ca^{++} concentration, no ciliary-type swimming was noted on the axonemes.

FLAGELLAR axonemes are cylindrical and consist of nine doublet microtubules surrounding a central pair of singlet microtubules. A radial spoke projects from each doublet toward the central sheath around the central pair.

Omoto and Kung (9, 10) showed central-pair microtubules to rotate 360° counterclockwise for each ciliary beat cycle in the case of *Paramecium*, and proposed a model in which central-pair rotation originates at the base of the cilium and regulates ciliary beating. Continuous rotation of central-pair microtubules in frayed flagella was observed by Kamiya (7) in the reactivated cells of *Chlamydomonas*. Omoto and Witman (11) also found that a tiny uniflagellate marine alga, *Micromonas pusilla*, had longer central-pair microtubules extended beyond the peripheral doublets. The longer central pair rotated continuously in one direction during flagellar movement. On the other hand, Tamm and Tamm (14) reported that the axis of each central pair of the compound comb plate cilia in ctenophore was always perpendicular to the bending plane. Based on ultrastructural observations, they concluded that the rotation of central-pair microtubules does not control the direction of bending movement of ciliary axoneme (13, 14).

We found central pairs to extrude from the elastase-digested axonemes of demembranated *Chlamydomonas* flagella with the addition of ATP, and reexamined the functions of central pair in flagellar movement.

Materials and Methods

Chlamydomonas reinhardii, a wild type (C-8), was grown in the culture medium of Sager and Granick (12) with slight modification. The cells were grown in 1.2 liters of medium and subjected to slower shaking (60 rpm) under constant illumination at 25°C. When the cell population reached 10^6 cells/ml, the cells were harvested, washed twice by centrifugation, and suspended in 10 ml of HMDS medium (30 mM Hepes, pH 7.5, 5 mM $MgSO_4$, 1 mM dithiothreitol (DTT), and 4% sucrose). Flagella isolation was carried out by the method of Bessen et al. (1); 2 ml of 25 mM dibucaine-HCl were added to the 10-ml suspension medium and mixed gently by pipetting for 3 min. After adding HMDS medium containing 0.5 mM EGTA, the isolated flagella were collected by centrifugation at 31,000 *g* for 20 min in the cold. The pellet was suspended in HMDEKP medium (30 mM Hepes buffer, pH 7.5, 5 mM $MgSO_4$, 1 mM DTT, 0.5 mM EDTA, 100 mM potassium acetate, and 0.5% polyethylene glycol [PEG], mol wt 20,000) containing 0.1% Nonidet P-40 for 4 min at 0°C. Axonemes were collected by centrifugation at 12,000 *g* for 10 min in cold, and gently suspended in 1 ml of cold HMDEKP medium.

Elastase digestion was based on the method of Brokaw (2). Axonemes were suspended in 1 ml of HMDEKP medium (170 μg/ml), and digested previously with 3 μg/ml elastase (Boehringer Mannheim Diagnostics, Inc. Houston, TX) for 120 s at room temperature. Digestion was terminated with 20 μg/ml chicken ovo trypsin inhibitor (Sigma Chemical Co., St. Louis, MO). Contamination by a small amount of trypsin on the elastase was previously prevented by addition of a soybean trypsin inhibitor (Sigma Chemical Co.). Presence of Ca ions from 10^{-9} to 10^{-3} M during digestion process had no effect on the bending movement, as described later. To bring about extrusion of the central pairs, the suspension of digested axonemes was mixed with an equal volume of the reactivation medium containing 1 mM ATP and a Ca buffer system, consisting of Ca and EDTA or EGTA at an appropriate ratio. Free Ca ion in the medium was calculated according to Goldstein (5). Concentration of Ca ion in the reactivation medium had no

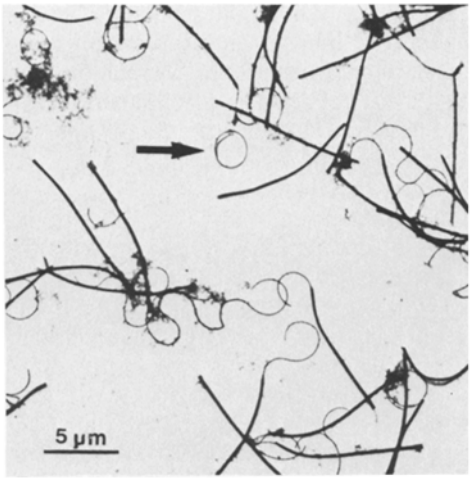

Figure 1. Extruded central pairs from the tips of *Chlamydomonas* axonemes. Arrow indicates the central pair which has extruded out in the medium. Electron micrograph. Bar, 5 μm.

effect on the fraction extruded (ratio of the number of the extruded axonemes to that of total axonemes).

Observations were made by Hitachi H-300 electron microscope, a Nikon phase-contrast microscope, and a dark-field microscope whose light source was an Ushio 100-W mercury arc lamp, as described previously (8). Images were recorded under the dark-field microscope on videotape with a high-sensitivity television camera (model No. CTC 9000; Ikegami, Tokyo, Japan) and videotape recorder (model No. J9; Sony, Tokyo, Japan), or under the phase-contrast microscope with stroboscopic illumination on Kodak Plus-X Pan film. Video images were reproduced and analyzed on a motion analyzer (model No. SVM-1110; Sony) connected with a video timer (model No. VTG-33; For A, Tokyo, Japan). The beat frequency was determined stroboscopically (i.e., measuring beat period required for one beat of axoneme in the reproduced images). The images were photographed frame by frame to 35-mm film.

Results

Our preliminary observations on the swimming behavior of reactivated *Chlamydomonas* flagella indicated that central-pair microtubules extruded occasionally from one end of the axonemes. To enhance the fraction extruded, we tried to digest demembranated flagella with elastase (4). Changing concentrations of elastase from 1 to 8 μg/ml in the axoneme suspension and the digestion time from 30, 60, 90, 120, and 150 s, we found the maximal fraction extruded to be obtained by digestion with 3 μg/ml elastase for 120 s at room temperature. In the present study, the addition of 400 μM to 1 mM ATP to demembranated *Chlamydomonas* flagella, after this digestion with 3 μg/ml elastase for 120 s was found to induce extrusion of the central-pair microtubules from the tips of more than 90% of the axonemes (Fig. 1). 0.2–0.8 mM ADP was also found to have the same effect, but 1–5 mM pyrophosphate was not.

By dark-field microscopy, it was seen that extrusion was either partial or complete; i.e., the length of the extruded part was 70–100% of the axoneme. The extruded central pair has already been reported to be a left-handed helix (7) (Fig. 2). Electron microscopy showed that the extruded part had a circular shape, with a uniform diameter of ~2.0 μm (Fig. 1). The end of the axoneme from which a central pair extruded was relatively slender, indicating that extrusion occurs from the flagellar tips.

After either partial or complete extrusion, axonemes continued to swim in the reactivation medium containing ATP. The swimming direction was always backwards, proximal end first with the extruded central pairs trailing like a tail from the flagellar tips (Figs. 2 and 3).

Demembranated, elastase-digested flagella often became stuck to the slide by their proximal ends. Perfusion of ATP under the cover slip caused the flagella to start bending. This was followed by progressive extrusion of central-pair microtubules from the other distal end of the axoneme. Even after the central pair had extruded into the medium, the reactivated flagella continued to bend, although the movements appeared somewhat irregular. The swimming speed of the flagella was slower than that of nonelastase-treated flagella. The mean frequency of the control flagella was 62.0 ± 1.3 Hz (n = 30 axonemes) at 10^{-9} M Ca ion and 67.7 ± 4.8 Hz (n = 30 axonemes) at 10^{-4} M Ca ion. Due to the rather irregular beating, various beat periods were required for beating of the axonemes after central-pair extrusion. The mean frequency was 10.4 ± 8.6 (Hz (n = 30 axonemes) at 10^{-9} M and 9.8 ± 6.3 Hz (n = 30 axonemes) at 10^{-4} M Ca ion.

As already reported (1, 6), demembranated *Chlamydomonas* flagella usually show either backward swimming with a symmetric bending pattern, or forward swimming with an asymmetric bending pattern, depending on the Ca^{++} concentration in the reactivation medium. A comparison of the bending behavior of axonemes from which central pairs had extruded, with that of control axonemes, showed that whereas the former generated a symmetric waveform in the reactivation medium containing either 10^{-9} or 10^{-4} M Ca ion, the latter exhibited Ca-dependent swimming behavior; an asymmetric ciliary type motion was noted at the lower Ca^{++} level (Fig. 4).

To confirm whether the loss of the central pair of an axoneme is responsible for the inability to generate asymmetric bending patterns, the time course of changes in bending patterns during digestion was examined. The axonemes digested with 3 μg/ml elastase for 30, 60, 90, or 120 s were reactivated in a medium containing 10^{-9} or 10^{-4} M Ca^{++} ions. The extrusion process of a central pair was so rapid that it occurred in less than a second. The numbers of extruding, asymmetric, and symmetric beating axonemes were counted. Table I shows the fraction extruded and ratio of the numbers of symmetric beating axonemes to the total number of beating axonemes. The fraction extruded increased, accompanied by an increase in the symmetric beating ratio, as shown in Table I. Some disagreement with the increases, such as a lower

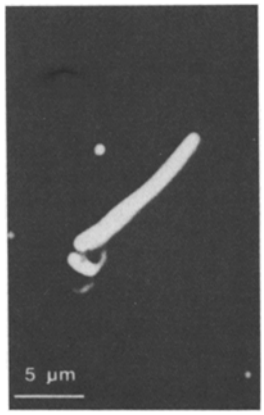

Figure 2. The helical structure of a central pair extruding from an axoneme. Dark-field micrograph. Bar, 5 μm.

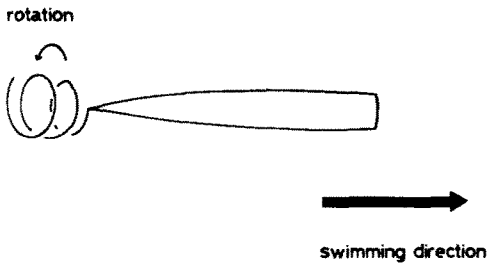

Figure 3. Schematic representation of the swimming direction (*thick arrow*) of an axoneme from which a central pair has been extruded, and the rotating direction of a central pair (*thin arrow*).

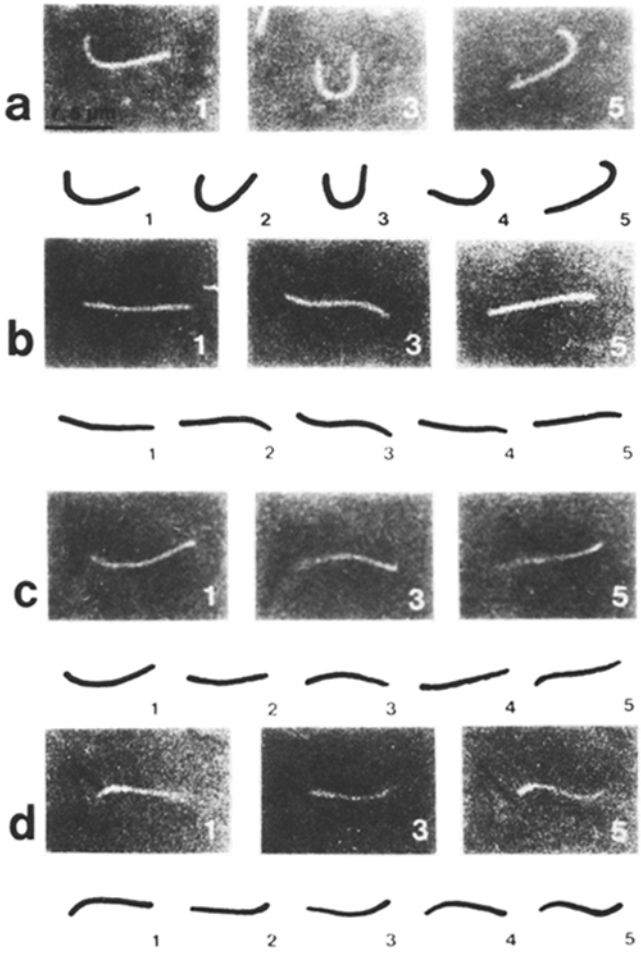

Figure 4. Stroboscopic recording of the sequence of bending movement of the axonemes from which central pairs have been extruded. Extrusion of the central pair with a length ~90% that of the axoneme was previously confirmed by dark-field microscopy observation. Phase-contrast micrographs. Tracing of the bending patterns appears beneath the photographs. Time interval of each tracing was 1/40 s. (*a*) Bending movement of a demembranated axoneme in the reactivation medium containing 10^{-9} M Ca ion. (*b*) Bending movement of a demembranated axoneme in the reactivation medium containing 10^{-4} M Ca ion. (*c*) Bending movement of a central-pair–extruded axoneme in the reactivation medium containing 10^{-9} M Ca ion. (*d*) Bending movement of a central-pair–extruded axoneme in the reactivation medium containing 10^{-4} M Ca ion. Time interval of each micrograph was 1/20 s. Bar, 7.5 μm.

fraction extruded rather than ratio of the symmetric beating axonemes, may be due to difficulty in distinguishing axonemes whose central pairs have extruded completley from those not extruding central pairs at the time symmetric beating axonemes were observed. In consideration of this, the fraction extruded may be said to be essentially consistent with the ratio of symmetric beating axonemes.

The bending patterns of axonemes digested with 3 μg/ml elastase for 120 s were compared before and immediately after central-pair extrusion. When reactivated in the medium containing a lower Ca^{++} concentration and ATP, the digested axonemes at first showed bending movement with asymmetric beating. After this bending, the central pairs started extruding progressively from the tips of the axonemes, causing at the same time the mode of bending of the axonemes throughout their length to change somewhat. The extrusion process was quite rapid, requiring less than a second. After extrusion, the axonemes started bending with symmetric beating patterns. The dark-field micrographs in Fig. 5 *a* show that the asymmetric beating pattern continued for ~1 s before extrusion. The bending form appeared to change somewhat when the central pair had extruded to a length exceeding 90% that of the axoneme, and finally took the form of symmetric beating. The extruding axonemes continued to bend with symmetric beating even when the Ca^{++} concentration of the medium was lower, as shown in Fig. 5 *b*. The same bending patterns as observed under a phase-contrast microscope are also shown in Fig. 4. Comparison of the bending patterns before and after central-pair extrusion, and the correlation between the fraction extruded and ratio of symmetric beating axonemes, indicate the axonemes to be quite likely incapable of generating asymmetric bending patterns after central-pair extrusion.

When axonemes with partially extruding central pairs swam in the reactivation medium, the left-handed helix of each central pair was noted to rotate continuously. The direction of rotation was counterclockwise, viewing the flagellum from the distal end, as described elsewhere (7). This rotation was evident, even when nearly the entire axoneme length adhered tightly to the slide, making bending motion practically impossible. Although the cause for rotating central pairs remains unclear at present, it does not appear to arise from rotation originating at the base of the axonemes (9, 10). Partially extruded central pairs rotated continuously, but were detached from the basal part, and in contact only with the distal part of the axonemes. Furthermore, we did not observe any motility of the central pairs extruded out in the reactivation medium containing ATP. From these observations, the

Table I. Time Course of Fraction Extruded and Ratio of Beating Patterns of Chlamydomonas Axonemes during Digestion with Elastase

Digestion time	Fraction extruded	Ratio of symmetric beating axonemes
s	%	%
30	44.1	55.4
60	57.1	76.5
90	75.3	89.5
120	90.2	92.3

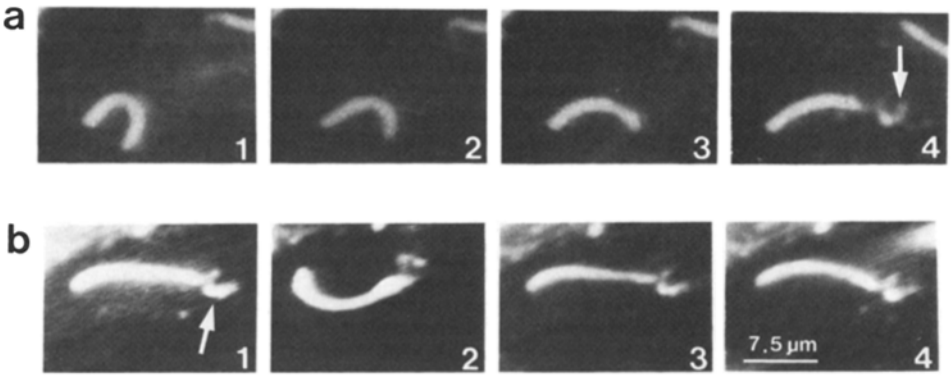

Figure 5. Video recording of the sequence of bending movement of the axonemes just before central-pair extrusion (*a*) or after the extrusion (*b*). Dark-field micrographs. Video images reproduced on a motion analyzer (model No. SVM-1110; Sony) were photographed frame by frame to 35-mm film. Bar, 7.5 μm. (*a*) Asymmetric bending movement of a demembranated axoneme in the reactivation medium containing 10^{-9} M Ca ions, just after perfusion of ATP to the axonemes. Time interval of each micrograph (*1*, *2*, and *3*) was 1/20 s, and the micrograph (*4*) was taken at 1 s after ATP perfusion. The extruding central pair with a length ~90% that of the axoneme (*arrow*). (*b*) Symmetric bending movement of the axoneme in the reactivation medium containing 10^{-9} M Ca ions, after extrusion of the central pair. Micrographs were taken at 2 s after ATP perfusion. Time interval of each micrograph was 1/20 s. The extruded central pair from the axoneme (*arrow*).

central-pair rotation appears to be due to interaction between radial spokes and central pair at present.

Discussion

The present results, together with previous work on the Ca-dependent swimming behavior and beat patterns of demembranated *Chlamydomonas* flagella (1, 6), showed that the backward swimming with symmetric beating of demembranated flagella may possibly be caused by the resting or "off" state of central-pair function. On the other hand, forward swimming with asymmetric beating may possibly be induced by the active or "on" state of central-pair function. Should this actually be the case, the functions of central pairs may be concluded as essential for the generation of asymmetric bending patterns. This conclusion seems to be consistent with the model proposed by Omoto and Kung (9, 10) for the beating of *Paramecium* cilia. They suggest that central-pair rotation may be essential to determine which of the nine peripheral doublets should slide actively during asymmetric ciliary beating. Although Ca ions are a possible candidate for switching on and off central-pair rotation, no differences in behavior of rotating central pairs could be found at lower or higher Ca concentrations at present, when the rotating central pairs extruded from axonemes were observed in the medium. Similar Ca^{++} independence of central-pair rotation has also been reported by Kamiya (7).

Changes in motility due to elastase digestion of demembranated flagella of sea urchin sperm have been reported by Brokaw (2). A gradual increase in bend angle and gradual decrease in beat frequency were noted during the process of digestion. Brokaw (2) mentioned that elastase digestion of interdoublet links may possibly induce changes in flagellar motility. A decrease in the beat frequency of digested and extruded axonemes was also noted in the present research, although nothing definite regarding this decrease can be said at present. It appeared also to be difficult to determine here whether elastase digestion causes central-pair extrusion or damage to other axonemal components. There may be the possibility that this digestion damages Ca-sensitive components, and this in turn may determine swimming direction and beating patterns. However, as described in Results, observations of initial bending patterns of the digested axonemes after ATP perfusion clearly show this possibility to be unlikely, and indicate the loss of rotating central pairs to be responsible for the inability to generate asymmetric bending patterns. The correlation between fractions extruded and ratios of beating patterns during elastase digestion appears to support this conclusion.

The presence or absence of Ca ions not only in the reactivation medium of the flagella model, but also in the extraction medium, affected the swimming direction and bending patterns, as also reported by Brokaw et al. (4). However, we could find no difference in the extrusion process and bending patterns observed here, when elastase digestion of axonemes was done in a medium containing 10^{-9}–10^{-3} M Ca ions.

A pf15 mutation has recently been reported to produce nonmotile flagella whose central pairs are missing (2). A recombinant of the paralyzed pf15 mutant and supressor mutant had motile flagella but still lacked the central pair. The flagellar bending pattern of this recombinant was essentially symmetric with large amplitude and thus was quite different from the wild-type pattern. According to Brokaw and Luck (3), the symmetric, large amplitude bending pattern may represent a "primitive" mode of bending, displayed by the recombinants lacking central pairs or radial spokes. They suggest that the function of "the central pair–radial spoke system" is conversion of the symmetric bending pattern into the asymmetric bending patterns required for efficient forward swimming. The modulating mechanism, i.e., the central pair–radial spoke system, may modify the bending pattern. The interpretation appears to be consistent with the on–off state of central-pair rotation proposed here, although discrepancy seems to remain between the forward mode beating in the recombinants and the backward mode beating in the central-pair extruded flagella. The details of the modulating mechanisms, what causes the central pair to rotate, and how various components in the system interact and generate an asymmetric bending pattern, remain to be elucidated.

We thank Dr. Y. Mogami of Ochanomizu University, Tokyo, Japan, for his technical advice and help throughout the study and Dr. S. Mihara of Institute of Applied Microbiology, University of Tokyo, for kind advice and supply of the strain, *Chlamydomonas*.

This research was supported in part by grants from the Ministry of Education, Science, and Culture of Japan.

Received for publication 30 July 1986, and in revised form 5 May 1987.

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