Direct Measurement of Inter-doublet Elasticity in Flagellar Axonemes

Itsushi Minoura\(^1\), Toshiki Yagi\(^2\), and Ritsu Kamiya\(^1\),* 

\(^1\)National Institute for Basic Biology, Okazaki 444-8385 and \(^2\)Department of Biological Sciences, Graduate School of Science, University of Tokyo, Tokyo 113-0033, Japan

**Abstract.** The outer doublet microtubules in ciliary and flagellar axonemes are presumed to be connected with each other by elastic links called the inter-doublet links or the nexin links, but it is not known whether there actually are such elastic links. In this study, to detect the elasticity of the putative inter-doublet links, shear force was applied to *Chlamydomonas* axonemes with a fine glass needle and the longitudinal elasticity was determined from the deflection of the needle. Wild-type axonemes underwent a high-frequency, nanometer-scale vibration in the presence of ATP. When longitudinal shear force was applied, the average position of the needle tip attached to the axoneme moved linearly with the force applied, yielding an estimate of spring constant of 2.0 (S.D.: 0.8) pN/\(\mu\)m for 1 \(\mu\)m of axoneme. This value did not change in the presence of vanadate, i.e., when dynein does not form strong cross bridges. In contrast, it was at least five times larger when ATP was absent, i.e., when dynein forms strong cross bridges. The measured elasticity did not significantly differ in various mutant axonemes lacking the central-pair microtubules, a subset of inner-arm dynein, outer-arm dynein, or the radial spokes, although it was somewhat smaller in the latter two mutants. It was also observed that the shear displacement in an axoneme in the presence of ATP often took place in a stepwise manner. This suggests that the inter-doublet links can reversibly detach from and reattach to the outer doublets in a cooperative manner. This study thus provides the first direct measure of the elasticity of inter-doublet links and also demonstrates its dynamic nature.

**Key words:** nexin/eukaryotic flagella/elasticity/Chlamydomonas/mutants/dynein

The ciliary and flagellar beating is based on the localized sliding movements between outer doublet microtubules, driven by a group of motor proteins, dynein (3, 5, 30, 34). Although this sliding mechanism is well established, it has not been made clear how the linear sliding movement of microtubules is converted into the cyclical bending of the axoneme. In particular, it is not known how microtubule sliding in an intact axoneme is limited within a certain length, and how the direction of sliding cyclically alternates. These questions should be fundamental to understanding how cilia and flagella beat.

An axonemal structure that has been regarded as essential to the sliding-to-bending conversion is the thin thread that connects the two adjacent outer doublets, called the nexin link or the inter-doublet link. This structure, identified by electron microscopy (6, 28, 38) and by thermal fractionation of axoneme (32, 33), has been assumed to provide the internal elasticity necessary for axonemal beating. Summers and Gibbons (34) have demonstrated that outer doublet microtubules undergo sliding when ATP is added to the axoneme that has been briefly treated with trypsin. Electron microscope observations have suggested that the trypsin treatment disrupts nexin links and radial spokes while leaving dynein arms almost intact. Thus the nexin links and radial spokes appear to be responsible for limiting microtubule sliding to certain range in intact axonemes (35). Of these, the nexin link, and not the radial spokes, must be especially important, since axonemes of certain *Chlamydomonas* mutants lacking radial spokes have been shown to be capable of beating (13, 29).

Despite the assumed importance of the inter-doublet link, the evidence that the adjacent outer doublets are in fact linked by elastic bridges has been only indirect. Brokaw (4) has found that the bend angle in the axo-
nemal waveform, and therefore the amplitude of inter-doublet shear, greatly increases after brief treatment of the axoneme with elastase, which is believed to digest elastic proteins. Also, Olson and Link (28) have observed by negative-staining electron microscopy that doublet microtubules in axonemes sprayed on electron microscope grids are connected by thin threads with variable lengths, suggesting that the outer-doublets may be cross-linked by highly elastic fibers. However, electron microscope observations also suggest that the inter-doublet links may be able to reversibly detach from and reattach to the outer doublet microtubules, under conditions where doublets can slide (2, 39). This indicates that the inter-doublet link does not need to be highly extendable for the adjacent microtubules to slide apart for a large distance of 0.1-0.2 μm, the amplitude of microtubule shear in beating axonemes. Finally, in an analysis of a high-frequency, back-and-forth vibration in the axoneme, Yagi and Kamiya (43) have obtained evidence that adjacent outer doublets in an axoneme can be displaced by as long as 0.1 μm by the elastic recoil of inter-doublet links. On an assumption that the force produced by dynein within an axoneme is 40 pN/μm (24), they have suggested that the spring constant of the inter-doublet links in the total axoneme is about 4 pN/mm for a 10 μm-long axoneme.

In the present study, we analyzed the elastic property of axonemes using a direct method. By applying forces to the axoneme longitudinally with a fine glass needle, we found that the axoneme displays a spring-like elastic behavior. The spring constant of the axoneme thus measured was compatible with the previous estimate. Interestingly, axonemes frequently displayed stepwise displacements, possibly reflecting the detachment and reattachment of the inter-doublet links.

Materials and Methods

Strains

*Chlamydomonas reinhardtii* 137 c (wild type) and the following flagellar mutants were used: *oda1*, lacking the entire outer-arm dynein (18); *idal*, lacking a subset of inner-arm dyneins (19); *pf14*, lacking the radial spokes; and *pf18*, lacking the central-pair microtubules (see ref. 10).

Axoneme preparation

Cells were grown in 200 ml of TAP medium (9) with aeration over a 12 h/12 h, light/dark cycle. Flagella were obtained by the method of Witman et al. (41). The isolated flagella were demembranated just before use by suspending them in HMDEKP (30 mM Hepes (pH 7.4), 5 mM MgSO₄, 1 mM dithiothreitol (DTT), 1 mM EGTA, 50 mM K-acetate and 1% polyethylene-glycol (MW: 20,000)) containing 0.1% Nonidet P-40.

Apparatus for observation

The apparatus used in this study was assembled essentially as designed by Tani and Kamimura (37). In brief, it is comprised of a horizontal microscope, a micromanipulator for holding a glass needle, and a position sensor for measuring the displacement of the needle. The output from the position sensor was amplified with a differential amplifier, monitored with an oscilloscope, and recorded with a digital data recorder (a modified audio-tape recorder DTC-59ESJ, Sony, Tokyo). The chamber containing the axoneme and the glass micro-needle was held with a pair of ULTRAlign micro-manipulators (561 series, Newport, Irvine, CA). The glass micro-needle was moved with a high-resolution actuator (ESA1330-OPT-1, Newport).

Stiffness measurement

For the measurement of the longitudinal stiffness of an axoneme, a flexible glass micro-needle was attached to the axoneme by the tip, and the base of the needle was moved by known distances in the direction parallel to the axonemal orientation (Fig. 1). The stiffness was measured from the shear deformation of the axoneme and the deflection of the glass needle. Axoneme was introduced into a flow chamber made of a stainless steel frame (1 mm-thick) and a pair of coverslips coated with poly-L-lysine (Sigma). After the introduced axonemal suspension was kept still for a few minutes, the chamber was perfused with HMDEKP to wash out the axonemes that did not attach to the glass surface. In most experiments, perfusion with HMDEKP containing 1 mM ATP was followed. After the perfusion, a glass micro-needle of about 0.5 μm diameter was attached to the center of an axoneme. The needle was produced with a glass needle-puller (PP-83, Narishige, Tokyo) and a microforge (MF-83, Narishige), and its spring constant was determined from the average of the

---

**Fig. 1.** Schematic drawing of the method used. For simplicity, the axoneme is drawn on an assumption that it splits into two groups of outer doublets when shear force is applied with the glass needle.
squared amplitude of its thermal vibration in water (14). The microneedle appeared to attach to the axoneme firmly when pushed lightly against the axoneme, as long as the surface of the needle was clean. To apply force on the axoneme, the glass needle was moved by the actuator in the direction parallel to the length of the axoneme.

The displacement of the needle base ($D$) was determined from the voltage applied to the actuator, and the displacement of the tip of the needle ($d$) was measured with a nanometer-scale accuracy by means of a diode position sensor (17). Stiffness of the axoneme ($k_a$) was calculated by the following formula:

$$k_a d = k_s (D - d),$$

where $k_s$ is the spring constant of the needle. For measurements in the presence of ATP, micro-needles with $k_s$ of 0.4–1.2 pN/nm were used. The spring constant of an axoneme was determined by the least-square fitting of the data obtained at more than five different magnitudes of force.

### Results

**Elastic properties of the wild-type axoneme**

To evaluate the longitudinal elasticity of axoneme, we measured the amount of the shear in the axoneme produced when force was applied in the direction of its length. For this purpose, a fine glass needle was attached to the middle portion of an axoneme by the tip, and the needle was then displaced by a known distance by means of an electric actuator. An important premise in this experiment is that the attachment between the axoneme and the glass needle, as well as that between the axoneme and the glass surface, is much stiffer than the axonemal elastic structure in question. This condition appeared to have been met, because the axonemal stiffness thus measured was in fact much greater in the absence of ATP, a condition where dynein arms form tight cross bridges (see below).

In the presence of 1 mM ATP, the needle tip attached to the axoneme frequently vibrated back-and-forth, at high frequency with a nanometer-scale amplitude (Fig. 2a, inset) (16, 27, 42). Upon application of external force by displacement of the needle, the average position of the vibrating axoneme moved in the direction of the applied force. The amplitude and frequency of the nanometer-scale vibration did not change even when external force was applied (Fig. 2a). The average position of the needle tip started moving within one second after the onset of force application and was settled at a new stable position within 2–10 seconds. In the experiment shown in Figure 2a, a force of 230 pN was applied and the needle tip moved about 11 nm. The needle tip moved back to the original position when the applied force was removed by reducing the voltage on the actuator (data not shown).

In almost all cases, the displacement of the needle tip was reasonably proportional to the applied force (Fig. 2b). Thus the longitudinal elastic component of the axoneme can be regarded as a Hookean spring. Its elastic limit appeared to be longer than 100 nm; at $\geq 50$ nm displacements, the movement of the needle often showed steps of several nanometers while still holding an overall linear relationship with the shear force (see below); and at $\geq 200$ nm displacements, the needle tended to detach from the axoneme. Spring constant of
a wild-type axoneme shown in Figure 2b, as determined by linear least-square fitting, was 22 pN/nm. The spring constant did not markedly depend on the direction of force; an almost identical value was obtained when the force was applied in the opposite direction (data not shown). Measurements with six axonemes yielded values of spring constant between 11 pN/nm and 40 pN/nm; for the length of 1 μm, the average ± standard deviation was 2.0 ± 0.8 pN/μm.

When experiments were performed in the absence of ATP, the elastic constant measured was much larger than that measured in 1 mM ATP. With a force smaller than 200 pN, as used in the measurement in the presence of ATP, no significant displacement of the glass needle was detected. Only with application of a greater force with a stiffer needle (2–5 pN/μm) did the needle tip show a displacement, which yielded an estimate of the spring constant to be about 100 pN/μm, i.e., five times larger than the value measured in the presence of ATP (Fig. 3).

The elastic constant of the axoneme remained low when measured in the presence of ATP and vanadate, which inhibits dynein ATPase by trapping it in a state in which dynein does not bind to microtubules strongly (7, 20, 26). Under these conditions, the axoneme did not display nanometer-scale oscillation. Upon application of external force, the needle tip was displaced as in the absence of vanadate (for example, see Fig. 5). The spring constant in this case is almost identical as that observed with axonemes displaying high-frequency vibration (Fig. 4).

Comparison of elasticity in mutant axonemes
Mutant axonemes lacking various internal components were next examined for their longitudinal elasticity.

Fig. 3. Determination of the longitudinal elasticity of axoneme in the absence of ATP. Relationship between the applied force and the displacement of the needle tip was shown. Experimental procedure was the same except that no ATP was contained in the medium, and a stiffer needle was used. Length of the axoneme: 12 μm.

Fig. 4. Comparison of elasticity in mutant axonemes. Spring constants were normalized by the length of the axonemes measured. In each column, the filled squares show the individual data from 3–7 axonemes, and the bar shows their mean value. One mM ATP was contained in the medium. WT, wild-type axoneme; V, wild-type axoneme measured in the presence of 20 mM vanadate; ida1, a mutant lacking the f subspecies of inner-arm dynein; oda1, a mutant lacking the outer-arm dynein; pf14, a mutant lacking the radial spokes; pf18, a mutant lacking the central-pair microtubules. All were measured in the presence of 1 mM ATP.

Figure 4 summarizes the data from those experiments as well as the data on the wild-type axoneme. The pf18 axoneme lacking the central-pair microtubules and the ida1 axoneme lacking a subset of inner-arm dynein displayed almost the same elasticity as that of the wild-type axoneme. On the other hand, the pf14 axoneme lacking the radial spokes and the oda1 axoneme lacking the outer dynein arm displayed somewhat smaller spring constants. However, due to the large scatter in the data, statistical test indicated that the difference in elasticity between these mutants and wild type cannot be taken as significant. From these results, however, it is confirmed that all the mutant axonemes examined have roughly the same order of elasticity as in the wild-type axoneme.

Stepwise displacement
In the majority of the experiments performed in the presence of ATP, the needle tip attached to the axoneme moved smoothly to a new stable position after application of shear force, as described above. However, it occasionally displayed jumps of several nanometers. The jump occurred mostly in the direction of the applied force and less frequently in the opposite direction, during the first 10 seconds after the application of force. Its occurrence cannot be controlled in our experiment, although it appeared to be more frequently observed at displacements greater than 30 nm. Jumps of the same magnitude were observed in the presence of vanadate (Fig. 5). No evidence has been obtained that
the jumps take some discrete lengths that reflect the size of axonemal components, such as tubulin.

Discussion

We have shown that the *Chlamydomonas* flagellar axoneme displays elastic deformation in response to forces applied in the longitudinal direction. To our knowledge, this is the first report of the detection and measurement of the longitudinal elasticity, which has long been postulated in theoretical studies (e.g., ref. 23). Together with the previously measured flexural rigidity of cilia and flagella (1, 15, 27), the data obtained in this study should be important for understanding the mechanics of axonemes.

It is likely that the observed longitudinal elasticity is due to some structures present between the outer doublet microtubules, since it increased several-fold when adjacent outer-doublets are tightly bound to each other under conditions where dyneins produce strongly bound cross bridges. That what we measured is the elasticity of the inter-doublet links is also supported by the observation that the mutant axonemes that lack various internal structures of axoneme retain the elasticity. However, since the measurements with the mutants pfl14 and odal1 yielded somewhat smaller spring constants, we cannot rule out the possibility that the radial spokes and outer dynein arms may also contribute to the elasticity. To verify this possibility, we will need further studies.

An important question in interpreting the data is how many inter-doublet links are involved in producing the observed elasticity. First of all, we have no data as to whether all the inter-doublet links along the entire length of doublet is evenly stretched by the externally applied force; more specifically, it is not known whether the links positioned close to the end of the axoneme are stretched to the same extent as those positioned close to the needle attachment site, or whether the links positioned in front with respect to the force direction are stretched like those positioned behind. Although this question remains to be answered by future experiments, here we assume that the force applied to the needle evenly stretches all the inter-doublet links between a pair of outer doublets, because the microtubule has been shown to be a markedly stiff structure (8). Secondly, it is not known how many pairs of outer doublets undergo sliding. In a simple case, a single axoneme may behave as if it were composed of two bundles of four or five outer doublets. In this case, the shear caused by the external force should take place between two pairs of outer doublets. In another extreme case, relative displacement may take place between all the adjacent pairs of doublets. In this case, the observed elasticity is that of a structure in which several groups of inter-doublet links are connected in series. Although we do not have enough information to decide on the exact number of the inter-doublet rows involved in the shear, we speculate that the axonemes under the present experimental conditions undergo shear displacement only along two pairs of outer doublets. This is because previous experiments on sliding disintegration of axonemes have indicated that the axoneme tends to disintegrate into two groups of outer-doublet microtubules in the presence of a high (>0.5 mM) concentration of ATP (22, 25, 36, 40), i.e., under the nucleotide conditions used in the present study. Thus we regard the observed elasticity is that of two rows of inter-doublet links arranged in parallel. This assumption should yield the lowest estimate for the spring constant of the links between two outer doublets. It must also be noted that the number of rows of inter-doublet links involved may not be constant even in the presence of a high concentration of ATP; it may differ in axonemes of different mutants, or even from one axoneme to another. Such a variation may cause a difference among the data with different mutants, as well as a large scatter in the data on a single species of axoneme.

Yagi and Kamiya (43) analyzed a special type of nanometer-scale oscillation that appears to be produced by the combined effect of the active force produced by dynein and internal elastic force. With an assumption that the axonemal dynein produces a shear force of 40 pN/μm, they estimated the spring constant of the axoneme, 10 μm long, to be 4 pN/nm. The estimate of the shear force was based on the maximal force measured in disintegrating sea urchin sperm axonemes (24). Our present data of elasticity, 22 pN/nm is five times greater than their value. However, recent studies suggest that
a single dynein can produce as much as 4–6 pN (12, 21, 31). Thus, the total dynein arms in the axoneme may be able to produce a shear force much greater than previously measured; if we consider only the outer arm, which is arranged at every 24 nm along the outer doublet, and assume that it produces 5 pN, the force produced by the total outer arms within a 1 μm-long outer doublet should be 5 pN × 1000/24 ≈ 200 pN, i.e., five times greater than the maximal force measured by Oiwa and Takahashi (24). Thus, it is possible that the force produced during the nanometer-scale vibration was higher than the assumed value, and the internal spring constant estimated (43) may have been several-fold less than the real value. These considerations suggest that the internal elasticity measured in this study is compatible with the previous estimate.

We have frequently observed that the shear displacement in the axoneme takes place in a stepwise manner. Such a movement cannot be accounted for by the force production by dynein since it is observed in the presence of vanadate, or even in the axonemes from which dynein has been removed by extraction with a high-salt concentration solution (data not shown). Thus this phenomenon must reflect the dynamic nature of the inter-doublet links. The observed abrupt movement could be accounted for by a cooperative folding/unfolding or, more plausibly, by cooperative attachment/detachment of the link. The observation that the stepwise movement occurs in both the direction of the applied force and the direction opposite to it indicates that the steps occur in a reversible process. Following the suggestions obtained from electron microscope observations (2, 39), we propose that such stepwise behavior is due to cooperative attachment/detachment of a subset of the links.

Ultrastructural studies have indicated that the inter-doublet links are present on the outer doublet at an interval of 96 nm (32, 39). Thus, if two rows of inter-doublet links are involved in our experiment, about 20 links should be responsible for the observed elasticity per 1 μm of axoneme. This consideration leads to an estimate of the spring constant of a single inter-doublet link to be 2 pN/nm/20 = 0.1 pN/nm. Assuming that a single inter-doublet link is as long as the wall-to-wall or center-to-center distance between adjacent doublets, 30–70 nm, this spring constant is about 15–35 times smaller than that of connectin (titin) of equivalent length (11). Collectively, the results of the present study suggest that the inter-doublet link is highly elastic and extendable, yet capable of reversibly attaching to and detaching from the outer doublets. Identification of the molecule that constitutes it remains one of the most important subjects in the study of axonemal motility.

Acknowledgments. The authors would like to thank Drs. Shinji Kamimura and Tomomi Tani for their help in designing the experimental system. I.M. has been a recipient of the Fellowship for Junior Scientists from the Japan Society for the Promotion of Science. This study has been supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan.

References
Inter-doublet Elasticity in Axoneme


(Received for publication, November 27, 1998 and accepted, November 30, 1998)