The Proximal Portion of Chlamydomonas Flagella Contains a Distinct Set of Inner Dynein Arms

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Abstract. A specific type of inner dynein arm is located primarily or exclusively in the proximal portion of Chlamydomonas flagella. This dynein is absent from flagella <6 μm long, is assembled during the second half of flagellar regeneration time and is resistant to extraction under conditions causing complete solubilization of two inner arm heavy chains and partial solubilization of three other heavy chains. This and other evidence described in this report suggest that the inner arm row is composed of five distinct types of dynein arms. Therefore, the units of three inner arms that repeat every 96 nm along the axoneme are composed of different dyneins in the proximal and distal portions of flagella.

Materials and Methods

Strains and Culture of Chlamydomonas Cells

Nomenclature and properties of the strains analyzed in this study are listed in Table I. Cells ~4 × 10^6 per 100-mm plates were cultured on solid medium at 25°C for 3 d under direct illumination from 34 watt fluorescent tubes that were positioned 3–5 in. from the culture dishes. Culture dishes were then transferred 3–5 ft from the light source and kept at 21°C for 16 h. Cells were collected in liquid medium at a concentration ~5 × 10^7 cells per ml, transferred under direct illumination and kept at 21°C for at least 2 h before further manipulation.

Solid medium was formed by 1.5% agar (Becton Dickinson & Co.) which was washed extensively with H_2O before use, and Sager's minimal medium (6) modified as follows: 10 ml of 10% sodium acetate-3H_2O, 2.8 ml of 8.3% MgCl_2-6H_2O and 0.2 ml instead of 3 ml of 10% MgSO_4-7H_2O were added per liter of medium. Labelling of cellular components was performed with 25 mCi of [35S]sulfuric acid per liter of medium. Liquid medium used for collecting the cells did not contain nitrate, acetate and [35S]sulfuric acid but contained all other ingredients at one-fifth of their original concentration.

Determination of Flagellar Length

Cells suspended in 0.2 mM SrCl_2, 0.01 M Hepes, pH 7.2 were fixed with 5 vol of 1% glutaraldehyde, 0.02 M phosphate buffer pH 7.4 and then analyzed by optic microscopy. At least eight photomicrographs were taken for each cell population. Direct measurements of flagellar length were performed on cell images projected on a screen. Magnification was 5,500.

Preparation of Flagella and Axonemes

All operations were performed at 5°C or 0°C. Cells were sedimented by centrifugation at 500 g for 10 min and resuspended in 0.2 mM SrCl_2, 0.01 M Hepes pH 7.2, 0.5 mg/liter leupeptin, 0.7 mg/liter pepstatin. These operations were repeated once. Cells were then exposed to pH 4 for 45 s by adding 0.5 N acetic acid in order to sever flagella from cell bodies. Restoration of pH 7.2 was obtained by adding 1 M KHCO_3.

To sever axonemes from cell bodies by exposure to NP-40 ~5 × 10^5 cells were suspended in 20 ml 5 mM Mg acetate, 50 mM Na acetate, 0.01 M Hepes pH 7.2, 0.5 mg/liter leupeptin, 0.7 mg/liter pepstatin. Then, an equal volume of the same solution also containing 0.07% NP-40 was added.
Table I. Chlamydomonas Strains Analyzed in this Study

| Strain | Flagellar phenotype* | Flagellar length* | Inner arm heavy chain deficiency | Linkage group of defective gene | Missing structure in the axoneme | Reference |
|--------|----------------------|-------------------|-------------------------------|--------------------------------|---------------------------------|-----------|
| 137(wild-type) | Abnormal motion | 12.0 ± 1.0(23) | | | | |
| pf28(oda2) | Paralyzed flagella | 9.8 ± 1.1(26) | 2,3' | XI | Outer arms | (15, 13) |
| pf15pf17 | Abnormal motion | 9.1 ± 1.3(53) | 2,3' | VI | Central complex, radial spokes | (19) |
| pf15pf17sup3 | Paralyzed flagella | 5.1 ± 1.1(49) | 2,3' | VI | Central complex, radial spokes | (11) |
| pf17 | Paralyzed flagella | 7.6 ± 1.5(35) | 1α,1β,2,3' | VII | Radial spokes | (10) |
| pf30pf28 | Paralyzed flagella | 3.3 ± 0.7(42) | 1α,1β,2,3' | XII, XI | Inner arm II, outer arms | (19) |
| pf23pf28 | Paralyzed flagella | 3.0 ± 0.4(14) | 1α,1β,2,3' | XI, XI | Inner arms, outer arms | (19) |
| pf22 | Paralyzed flagella | 4.4 ± 0.9(41) | 2,3,3' | I | Inner arms, outer arms | (9) |

* Flagellar phenotypes and lengths were observed on cells cultured in the presence of [35S]sulfuric acid as described in Materials and Methods.
† Number of determinations is reported in parentheses. Flagellar length is expressed as an average ± SD. Cells not bearing flagella were not accounted for.

and mixed rapidly. Axonemes were severed by the same procedure also in the absence of Mg and Na acetate.

Flagella or axonemes were isolated by differential centrifugations at 500 g for 15 min and sedimented at 30,000 g for 30 min. NP40 to the final concentration of 0.1% was added before the final centrifugation of axonemes that were severed by exposure to either pH 4 or 0.035% NP-40. Supernatants of axonemes prepared by subsequent exposure to 0.035% NP-40 and 0.1% NP-40 in the absence of Mg and Na acetate were concentrated by centrifugation on centricon 30 (Amicon Corp., Danvers, MA).

Regeneration of Flagella

A 200-ml suspension of 35S-labeled flagella (10⁶ cells/ml) was processed by the pH shock method to sever flagella at first time. Then, cell bodies were suspended in the original volume of medium and exposed to light at 25°C for 60 min to regenerate flagella to the extent of their full length. During that time, 50-ml aliquots of regenerating cell bodies were processed again by the pH shock method at 15, 45, 60, and 75 min after the first deflagellation. For this purpose, the cell bodies were collected at 0, 15, 30, and 45 min from the time of suspension of the cell bodies in medium because each sequence of centrifugation and resuspension of cells in medium or in the solution used for the pH shock was performed in 15 min.

Electrophoresis and Densitometry of Axonemal Components

Electrophoresis of dynein heavy chains was performed as described (19). That procedure resolves inner dynein arm heavy chains in six electrophoretic bands referred to as 1α, 1β, 2, 3, 3', and 3".

Independent electrophoreses of the same sample of flagella resulted in identical electrophoretic patterns of the six inner arm heavy chains. In contrast, electrophoreses of independent preparations of flagella from the same Chlamydomonas strain sometimes result in electrophoretic patterns showing a reduction of component 3 (Fig. 1). This apparent deficiency of component 3 depends on the amount of flagella that are present in the sample analyzed. Evidence supporting this interpretation is reported in Results.

An autoradiogram of the gel portion containing the dynein heavy chains and a major membrane component shows that the inner arm heavy chains of the mutant pf28 are resolved into six electrophoretic bands referred to as 1α, 1β, 2, 3, 3', and 3". The amount of heavy chains 1α and 1β are higher than that of 2 and 3 and lower than that of 2 and 3 as indicated by the relative intensities of the corresponding electrophoretic bands. The ratio between heavy chains 1α and 1β of 0.94 ± 0.05 (SD, n = 4) and the ratio between the same heavy chains together and all the others (i.e., 2, 3, 3', and 3'') of 0.47 ± 0.07 (SD, n = 4). Heavy chains 1α and 1β are subunits of the inner arm II whereas 2 and 3 and 3' and 3'' are components of inner arm I2 and 13, respectively (19).

Heavy chains 3' and 2 are respectively absent and
deficient, in strains pf15pf17 and pf15pf17sup3, whereas
they are present at similar concentrations in flagella of the
mutants of pf28 and pf17 (Fig. 1). Therefore, the deficiency
of heavy chains 3' and 2 is correlated with a flagellar length
< 6 μm and not with the absence of flagellar motility (Table
I). The same correlation was observed with the analysis of
a variety of short flagella mutants or recombinant strains (19)
that are defective for different axonemal substructures (data
not shown) including different types of inner arms (see last
section of Results).

Heavy chain 3' and 2 may be formed in situ or transported
in the flagellar space after that flagella reach a certain length.
This aspect of flagellar morphogenesis was investigated fur-
ther through the analysis of regenerating flagella.

One Inner Arm Heavy Chain Is Assembled during the
Second Half of Flagellar Regeneration

To determine whether or not all inner arm heavy chains are
assembled at the same time during the formation of flagella
we analyzed inner arm heavy chains of the mutant pf28
flagella at different stages of flagellar regeneration. First har-
vests of long flagella, which were obtained in order to induce
the regeneration, were adopted for comparison (Fig. 2) first
and last lanes.

Flagellar stubs formed after 30 or 45 min of regeneration
lack the inner arm heavy chain 3', are deficient for heavy
chain 2 and contain heavy chain 3 at the highest concentra-
tion (Fig. 2). Flagella > 6 μm long and collected after 60 and
75 min of regeneration contain increasing amounts of heavy
chains 1α and 1β, 2', 2, and 3' relatively to heavy chain 3.
Heavy chain 3' and 2 increase more than the others. The ratio
between heavy chains 1α and 1β together and all the others
is close to 0.5 in every sample analyzed during the regener-
ation.

The majority of heavy chain 3' and 2 are incorporated last
into the axoneme. However, the evidence does not provide
information about the assembly site of these inner arm heavy
chains in the axoneme because two-thirds and one-third of
axonemal proteins are added respectively at the distal and
proximal part of elongating flagella (25). The identification
of the assembly site of inner arm heavy chain 3' and 2 was
achieved through the analysis of partially extracted axo-
memes.

A Subset of Inner Arms Is Extracted Selectively from
the Axonemes

We pursued the analysis of partially extracted axonemes in
view of the possibility that inner arms present specifically at
one or the other end of the axoneme could be solubilized
differentially. For this purpose we first followed a procedure
allowing the isolation of axonemes that can be reactivated in
respect to motility (2). Then we altered the procedure by
lowering the ionic strength because we observed that under
those conditions the axonemes are opened at one end and ex-
tracted partially.

We prepared flagella or axonemes from four aliquots of the
same culture of the mutant pf28 following procedures in-
volving a deflagellation by pH shock or exposure to NP-40
in the presence or absence of Mg and Na acetate. Then we
compared the electrophoretic patterns formed by inner arm
heavy chains that were present in each sample.

The comparison of samples prepared either by pH shock
or by NP40 in the presence of Mg and Na acetate shows that
heavy chains are present in both flagella and axoneme sam-
ples at similar concentrations, with the exception of heavy
chain 3 that is present in reduced amounts in axonemes (Fig.
3), first three lanes. In contrast, axonemes prepared by ex-
posure to NP40 in the absence of Mg and Na acetate retain
all inner arm heavy chain 3', lack heavy chain 2' and 3, and
have reduced amounts of 1α and 1β and 2 (Fig. 3), fourth
lane. Intensity ratios of heavy chains 1α, 2, and 3' relative
to heavy chain 1α are 1, 3.2, and 4.5, respectively.

Soluble proteins extracted from the last sample of axo-
memes at the time of deflagellation include the complement
of inner arm heavy chains 1α, 1β, 2', 2, and 3 but no trace
of heavy chain 3' (Fig. 3), last two lanes. Therefore, each
heavy chain maintains its electrophoretic mobility unaltered
even after a solubilization process from whole and motile
cells.

The solubilization of heavy chains occurred within sec-
onds after the exposure of NP-40 because subsequent and
rapid addition of Mg and Na acetate did not prevent or alter
the extraction of heavy chains. In addition, the solubilization
required specific ionic conditions because the exposure to
NP-40 in the absence of 0.01 M Hepes, pH 7.2 prevented the
extraction (data not shown).

Selective solubilization of heavy chains 1α, 1β, 2', 2, and 3
occurred also in preparations of wild-type axonemes obtained
by exposure to 0.035% NP-40 in the absence of Mg and Na
acetate (Fig. 4) third lane. In those samples outer dynein arm
heavy chains were extracted as well as the subset of inner
arm heavy chains. Therefore, the retention of a subset of in-
nner arm heavy chains including 3' is specific and limited to a
small percentage of inner dynein arms. This evidence suggested that the location of the insoluble inner arms within axoneme could be identified by electron microscopic analysis.

**A Distinct Set of Inner Arms Is Located in the Proximal Part of the Axoneme**

We first analyzed by negative staining the axonemes of the mutant *pf28* that were isolated by the NP-40 method in the presence or absence of Mg and Na acetate. Axonemes prepared in the presence of Mg and Na acetate appear intact in their structure (Fig. 5 a). In contrast, axonemes isolated in the absence of Mg and Na acetate were splayed for approximately two-thirds of their length leaving single or groups of outer double microtubules free at one end of the axonemal...
structure (Fig. 5 b). Splayed axonemes that were analyzed at higher magnification appeared to lack the central microtubules. These central microtubules usually were found close to the axoneme (data not shown).

Electron micrographs of thin cross-sections of splayed axonemes showed that the unopened end of the axonemal structure is the proximal portion. All sections (n = 15) where the axonemal structure appears to be closed or opened in one to five points between adjacent outer doublets have a structure called “beak” inside the B tubule of outer doublets 5 and 6. That structure is present only in the proximal part of the axoneme (7) (Fig. 6, a-f). In contrast, cross sections of outer doublets completely separated from each other do not contain the “beaks” (Fig. 6, g, h, and i). The central microtubules were seen in 43% of this last type of section (n = 30) (Fig. 6, h and i) and not in the others.

The inner arms, identified as 18-nm-long projections located between radial links and radial spokes (9) were found in association with at least 70% of the outer doublets in cross-sections (n = 15) of the proximal portion of the axoneme. In contrast, they were found to be associated at the most with 16% of the outer doublets in cross-sections (n = 30) of the distal portion of the axoneme. Therefore, the majority or all inner arms formed by heavy chain 3' and the insoluble subset of heavy chains 1α, 1β, 2 and 3 are located in the proximal portion of flagella. On the other hand, the inner arms formed by soluble heavy chains 1α, 1β, 2', 2, and 3 are extracted from medial and distal parts of flagella.

The inner arms present in cross-sections where some of the outer doublets are not connected by nexin links are associated with outer doublets without any apparent order relative to the location of the break between doublets. Moreover, breaks between doublets occurred in different positions relative to outer doublets 1 and 2 (these are connected by a bridge other than inner or outer arm [7]), (Fig. 6, d-f). Therefore, the extraction of inner arms occurring at the opening of the axonemal structure affects middle and distal regions of the axoneme and not a subset of specific outer doublets along the axoneme.

Inner Arm Mutants Are Defective for Different Subsets of Heavy Chains 2', 2, and 3

Combined biochemical and electron microscopic analyses of the mutants pf30 and pf23 led us to identify heavy chains 2' and 2 as components of the inner arm 12 that is distal to radial spoke S2 (19). In contrast with that interpretation, the

Figure 4. Electrophoretogram of 35S-labeled flagellar polypeptides. First and second lane: axonemes of the mutant pf28 severed by exposure to 0.035% NP-40 in the presence of 0.01 M Hepes, pH 7.2 and proteolysis inhibitors and by the pH shock method, respectively. Third lane: axonemes of wild-type cells severed by exposure to 0.035% NP-40 in the presence of 0.01 M Hepes, pH 7.2 and proteolysis inhibitors. Equal amounts of radioactivity were analyzed in each lane. Bands referred to as 1α, 1β, 2, and 3' are indicated at the left side.

Figure 5. Electron micrographs of mutant pf28 axonemes prepared by negative staining. (a) Axonemes severed by exposure to 0.035% NP-40, 5 mM Mg acetate, 50 mM Na acetate 0.01 M Hepes, pH 7.2. (b) Axonemes severed by exposure to 0.035% NP-40, 0.01 M Hepes, pH 7.2. Bar, 10 μm.
The lack of heavy chain 2 does not necessarily involve the lack of heavy chain 2' or the deficiency of heavy chain 3 because flagella of the strain pf22 and pf23pf28 lack heavy chain 2 but are not deficient for heavy chain 2' or 3, respec-
In addition, pf22, pf23, and pf30, mutations affect a different subset of inner arm heavy chains \(2', 2, \text{ and } 3\). This evidence suggests that each heavy chain \(2', 2, \text{ and } 3\) forms a distinct inner arm. A model of the organization of the inner arm row is shown in Fig. 8. This model is described in the following section.

Discussion

Organization and Polarity of the Inner Dynein Arm Row

We proposed previously (19) a model of the organization of the inner arm row of \textit{Chlamydomonas} axonemes that includes the following features. First, the row is formed by three inner arms repeating every 96 nm. Second, each inner arm, I1, I2, and I3, is composed of two heavy chains referred to as 1\(\alpha\) and 1\(\beta\), 2 and 2' and 3 and 3', respectively. Finally, inner arms I2 and I3 are more similar to each other than to inner arm I1.

The above model, however, could not provide an explanation for the following observations. Long and motile flagella from wild-type and mutant strains have six inner arm heavy chains whereas short and immotile flagella from mutants lack heavy chain 3' and part of heavy chain 2. Moreover, wild-type flagella have reduced amounts of heavy chains 2' and 3' relative to the amounts of heavy chains 2 and 3.

To explain these observations we considered that heavy chains 2 and 3 may be converted into 2' and 3', respectively, by a posttranslational modification that depends on motility and length of flagella. Alternatively, heavy chain 3' and 2 may be missing and deficient, respectively, in immotile and short flagella because they are located in a part of the axoneme that is not assembled in short flagella. We obtained evidence in agreement with the second hypothesis by the analysis of the molecular composition of inner arms located in the proximal region of the axoneme. Those arms contain heavy chains 3' and 2', differ from inner arms of middle and distal regions and are missing in short flagella.

We deduce that each inner arm I2 and I3 is composed of two heavy chains because the ratio between both heavy chains of inner arm I1 and all heavy chains of inner arm 12 and 13 is close to 0.5 during flagellar regeneration and in full length pf28 flagella. In addition, inner arms I2 and I3 may be composed of identical subunits because inner arms remaining in partially extracted axonemes are composed uniquely of heavy chains 2 and 3' and 1\(\alpha\) and 1\(\beta\). The same hypothesis is supported also by the analysis of the mutant pf22 that lacks heavy chain 2 and is deficient for heavy chain 3. Electron microscopy of longitudinal sections of pf22 axonemes (Piperno, G., E. Smith, and W. Sale, unpublished observations) showed that the 96 nm repeats of inner arms lacked either inner arm I2 or I3 depending on the section under analysis. Therefore, the defect of heavy chains 2 and 3 corresponds to the defect of inner arms I2 and I3.

This last piece of evidence also suggests that inner arm triplets have different compositions depending on their positions along the axoneme in agreement with the fact that inner arms in the proximal region contain heavy chains 2 and 3' and not heavy chains 2' and 3. Heavy chains 2' and 3 may
be located exclusively at the distal region of the axoneme because they are present together with heavy chains 1α and 1β and 2 in short stubs of regenerating flagella and increase less than heavy chain 2 and 3' during elongation and completion of the axoneme structure.

Heavy chains 2' and 3, however, form the same inner arm triplet only in part of the axoneme because heavy chain 3 is present at a concentration higher than that of heavy chain 2'. Therefore, heavy chain 3 may extend over both distal and middle regions of the axoneme. For the same reason, heavy chain 2 which has a concentration higher than that of heavy chain 3' may extend over both proximal and middle regions, whereas heavy chain 3' is only proximal.

Together, these observations and hypotheses suggest the addition of three details to the model of the organization of the inner arm row already described. First, each inner arm I2 and I3 is formed by two identical subunits. Therefore, two types of inner arms I2 and I3 are assembled in the axoneome. Second, the heavy chains 3' and 2', which are present in the axoneme at a concentration lower than that of heavy chains 3 and 2, are located in proximal and distal portions of the axoneme, respectively. Finally, heavy chains 3 and 2 are located in distal and middle regions and in proximal and middle regions of the axoneme, respectively. A schematic representation of this last model of the inner arm row is shown in Fig. 8.

The row of outer dynein arms is formed by one single type of dynein repeating every 24 nm. In contrast, the inner arm row has two types of structural polarity. First, the polarity created by different arms in each triplet repeating every 96 nm. Second, the polarity created by a specific disposition of different triplets of arms along the axoneme. Each element of complexity in the inner arm row may serve a different function. While different proximal inner arms may participate in mechanism initiating axoneme bending waves, different distal inner arms and the polarity within inner arm triplets may provide the regulation needed to create asymmetric or symmetric flagellar bends during ciliary or flagellar kinds of motion (3).

**Function and Molecular Composition of Inner Arms Located in Different Regions of the Axoneme**

The existence of a difference between the mechanism of bend initiation and the mechanism of bend propagation was suggested by the analysis of sperm flagella that were stimulated to move under different conditions (23, 4). The same difference may exist between initiation and propagation of Chlamydomonas flagella bending patterns. On this basis the inner arm heavy chain 3' may be one of the molecules responsible for initiating the principal bend within flagella >6 μm long.

Heavy chain 3' is present only in flagella >6 μm long and is the only dynein of the inner arm I3 located in the proximal region. Moreover, the presence of heavy chain 3' among the outer arm-less mutants differentiates the oda mutants, which are motile, from the mutant pf13A, which is paralyzed (11, 19). The mutant pf13A becomes motile only after prolonged growth on minimal medium and agar (3) and in coincidence with the assembly of heavy chain 3' within the axoneme (Piperno, G., unpublished). On the other hand, the initiation of a principal bend occurs also in recombiant strains with short flagella and lacking heavy chain 3'. Therefore, the function of that heavy chain under anomalous conditions may be bypassed by one or several types of dynein arms working together.

Outer arms or inner arm II, which are present in the proximal region, are not necessary for the initiation of the principal bend because the mutants pf28 and pf30 are motile and are lacking the outer arms and the inner arm II, respectively (3, 19). Other putative components of the proximal region of the axoneme may control the waveforms of flagella but not the initiation of the principal bend because they affect the ciliary but not the flagellar kind of motion. Those components, including a polypeptide defective in the mutant pf23 (9), were found to be missing in mbo mutants, mutants moving only with flagellar motion. Some of them may be located in the proximal region of the axoneme in the structure present within the B tubule of outer doublets 5 and 6 (22).

**Inner Arms of the Proximal Region and Control of Flagellar Length**

Several observations have indicated that the length of Chlamydomonas flagella is under active control. First, cells regenerate one or both flagella to their original length after the amputation of primary flagella (20, 21). Second, cells that resorb their flagella in response to a stimulus usually regenerate their flagella to the original length when the stimulus is removed (14). Finally, temporary dikaryons obtained by mating short-or-long flagella mutants to wild-type cells may adjust all four flagella to wild-type length.

The existence of a molecular mechanism controlling flagellar length in Chlamydomonas is very likely but the components of this system remain to be identified. Some of the molecules forming the axoneme may limit the extent of flagellar growth through the size of their intracellular pool or the rate of their assembly. In this case, the study of the regulation of flagellar length may be directed to one or a few of the several hundreds of axonemal proteins.

Molecular components of proximal inner arms I2 and I3 may participate in the regulation of flagellar length for the following reasons. First, proximal inner arms I2 and I3 are lacking in all the mutants with flagella <6 μm long independently from their motility. Second, proximal inner arms I2 and I3 are assembled in the axoneme in the last phase of flagellar regeneration. Finally, the differentiation of the proximal part of the inner arm row does not have a parallel in other substructures that are assembled along the axonemes such as, outer dynein arms, radial spokes or central pair complex.

The molecular composition of proximal inner arms I2 and I3 is known only in part because mutants lacking specifically one of these structures have not yet been identified. In addition molecular complexes formed by heavy chains 3' and 2 have not been isolated; heavy chains 3' and 2 so far copurify with heavy chains 2' and 3 and four other polypeptides with lower molecular weight including actin and the Ca²⁺-binding protein caltractin/centrin (19) (Piperno, G., unpublished).

In summary, our initial observation that the set of inner arm heavy chains of short and paralyzed flagella differs from that of long and motile flagella has led us to modify the model of the organization of the inner arm row by postulating a distinction between inner arms of the proximal, middle and distal regions of the axoneme. Inner arms located in the proximal region may participate in the regulation of flagellar length and initiate the principal bend of flagella. Investigations on the molecular composition, synthesis and assembly
of proximal inner arms may provide further support to these hypotheses.

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