

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.

INNER and outer dynein arms are motile structures that generate the sliding of adjacent outer doublet microtubules within the axoneme of cilia and flagella. Both inner and outer arms participate in complex regulatory mechanisms converting microtubule sliding into axonemal bending. However, they have different functions. The absence of one type of inner arm from axonemes of *Chlamydomonas* mutants alters mainly the waveforms of flagella whereas the absence of outer arms reduces the beat frequency (3, 13).

Inner dynein arms differ from outer arms in their structure (5) and molecular composition (9, 16). At least three types of inner arm compose a unit which is repeated with a periodicity of 96 nm in a row along the axonemes of *Chlamydomonas* flagella (19). The inner arm referred to as I1 is proximal to radial spoke S1, whereas the inner arms I2 and I3 are distal to spokes S1 and S2, respectively. Each inner arm is composed of two heavy chains, each having a site for ATP hydrolysis as well as a variety of intermediate and light chains including actin (17, 18). In contrast, only one type of outer dynein arm repeating every 24 nm forms a row parallel to that of the inner arms. Each outer arm is composed of three heavy chains, two intermediate and several light chains.

Immotile and short flagella of several *Chlamydomonas* mutants appear to be deficient for two heavy chains each forming inner arms I2 and I3, respectively (19). This deficiency may result from the lack of posttranslational processing of inner arms I2 and I3 heavy chains or is a consequence of defective assembly or synthesis of one or more axonemal components.

To test these hypotheses we have analyzed inner arm heavy chains from various mutants defective for either motility or length of their flagella. Results of our investigation show that flagella < 6 μm long are defective for two inner arm heavy chains. Each of these heavy chains forms one type of arm that is located in the proximal portion of the axoneme.

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 $\sim 4 \times 10^6$ 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 $\sim 5 \times 10^7$ 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 H₂O before use, and Sager's minimal medium (6) modified as follows: 10 ml of 10% sodium acetate·3H₂O, 2.8 ml of 8.3% MgCl₂·6H₂O and 0.2 ml instead of 3 ml of 10% MgSO₄·7H₂O 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 SrCl₂, 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 SrCl₂, 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 KHCO₃.

To sever axonemes from cell bodies by exposure to NP-40 $\sim 5 \times 10^9$ 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
		μm				
137(wild-type)		12.0 \pm 1.0(23)†				
<i>pf28(oda2)</i>	Abnormal motion	9.8 \pm 1.1(26)		XI	Outer arms	(15, 13)
<i>pf15pf17</i>	Paralyzed flagella	6.0 \pm 1.3(53)	2,3'	III, VII	Central complex, radial spokes	(1,9)
<i>pf15pf17sup_{pf3}</i>	Abnormal motion	5.1 \pm 1.1(49)	2,3'	III, VII, VI	Central complex, radial spokes	(11)
<i>pf17</i>	Paralyzed flagella	7.6 \pm 1.5(35)		VII	Radial spokes	(10)
<i>pf30pf28</i>	Paralyzed flagella	3.3 \pm 0.7(42)	1 α ,1 β ,2,3'	XII, XI	Inner arm II, outer arms	(19)
<i>pf23pf28</i>	Paralyzed flagella	3.0 \pm 0.4(14)	1 α ,1 β ,2',2,3'	XI, XI	Inner arms, outer arms	(19)
<i>pf22</i>	Paralyzed flagella	4.4 \pm 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 \pm 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 cells (10⁸ cells/ml) was processed by the pH shock method to sever flagella a 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 cells were processed again by the pH shock method at 30, 45, 60, and 75 min after the first deflagellation. For this purpose, they 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 1 α , 1 β , 2', 2, 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 results in electrophoretic patterns showing a reduction of component 3 (See Fig. 2, first and last lane as an example). This apparent deficiency of component 3 depends on the amount of short flagella that are present in the sample analyzed. Evidence supporting this interpretation is reported in Results.

Autoradiography and staining by silver of inner arm heavy chains result in electrophoretic patterns that are undistinguishable. Therefore, the autoradiography of electrophoretic bands followed by densitometry allowed for an estimate of relative amounts of inner arm heavy chains.

Scanning of images was performed with a Microtek scanner connected to a Macintosh IICx computer. Processing of images and measurement of peak intensity was performed by Image 1.28c software. Intensity of each peak under analysis increased linearly with time of exposure of the film.

EM

EM of thin sections and negatively stained samples was performed as described (9, 24).

Results

Short Flagella of Different Mutant Strains Lack One Inner Arm Heavy Chain

We observed that short and paralyzed flagella from mutant strains of *Chlamydomonas* carry five inner dynein heavy chains instead of the six which were identified in wild-type flagella (19). To determine whether flagellar length or paralysis or both are correlated with the number of inner arm heavy chains we have analyzed the strains *pf28*, *pf15pf17*, *pf15pf17sup_{pf3}* and *pf17* that differ for the length or motility of their flagella (Table I). The comparison of the strains was performed by electrophoresis of ³⁵S-labeled flagellar proteins of molecular weight close to 400,000 (Fig. 1). Flagella instead of axonemes were adopted for the analysis to avoid losses of dynein arms that may occur after the solubilization of the flagellar membrane.

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 1 α , 1 β , 2', 2, 3, and 3' (Fig. 1). The amounts of heavy chains 1 α and 1 β 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 1 α and 1 β is 0.94 \pm 0.08 (SD, n = 4) and the ratio between the same heavy chains together and all the others (i.e., 2', 2, 3, and 3') is 0.47 \pm 0.09 (SD, n = 4). Heavy chains 1 α and 1 β are subunits of the inner arm II whereas 2' and 2 and 3 and 3' are components of inner arm I2 and I3, respectively (19).

Heavy chains 3' and 2 are respectively absent and

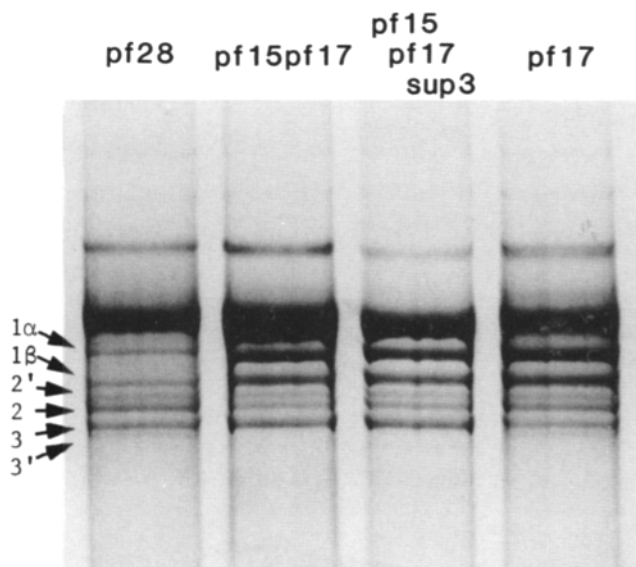


Figure 1. Electrophoretograms of ^{35}S -labeled flagellar polypeptides of the mutant *pf28*, the recombinant strains *pf15pf17* and *pf15pf17sup₃* and the mutant *pf17*. Equal amounts of radioactivity were analyzed in each lane. Bands referred to as 1α , 1β , $2'$, 2 , 3 , and $3'$ are indicated at the left side. The intense, wide band present in each electrophoretogram is a flagellar membrane protein. The two intense bands present in the last three lanes below the membrane protein are formed by both outer and inner arm heavy chains (19).

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 $< 6 \mu\text{m}$ 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 further 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 harvests 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 concentration (Fig. 2). Flagella $> 6 \mu\text{m}$ 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 regeneration.

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 axonemes.

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 extracted partially.

We prepared flagella or axonemes from four aliquots of the same culture of the mutant *pf28* following procedures involving 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 samples 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 exposure 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 1β , 2 , and $3'$ relative to heavy chain 1α are 1, 3.2, and 4.5, respectively.

Soluble proteins extracted from the last sample of axonemes 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 seconds 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 inner arm heavy chains including $3'$ is specific and limited to a

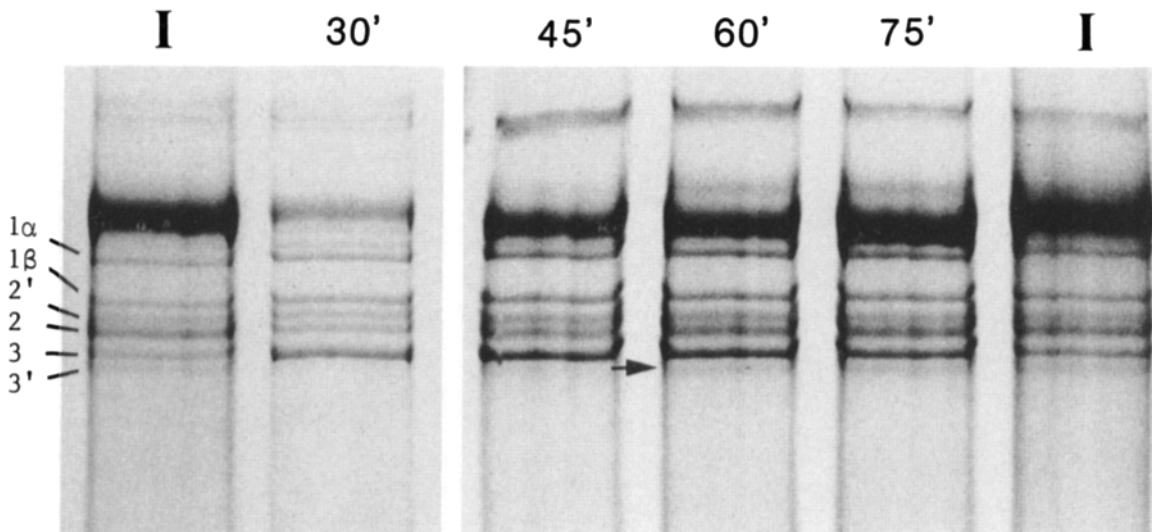


Figure 2. Electrophoretograms of ³⁵S-labeled flagellar polypeptides of the mutant *pf28*. First and last lanes: flagella severed a first time from two independent cultures of cells. Second, third, fourth and fifth lanes: regenerating flagellar severed after 30, 45, 60, and 75 min, respectively, from the time of the first deflagellation. Equal amounts of radioactivity were analyzed in each lane. Bands referred to as 1α, 1β, 2', 2, 3, and 3' are indicated at the left side. Arrowhead indicates the heavy chain 3'.

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

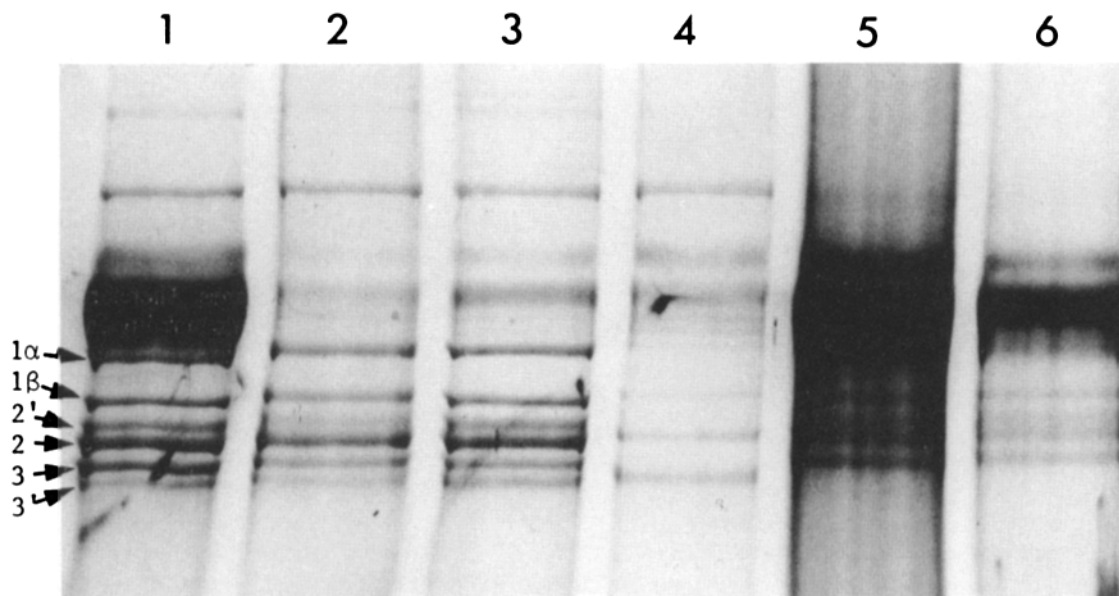


Figure 3. Electrophoretograms of ³⁵S-labeled flagellar polypeptides of the mutant *pf28*. First lane: flagella severed by the pH shock method. Second lane: axonemes prepared from flagella severed by the pH shock method. Third lane: axonemes severed by 0.035% NP-40 in the presence of 5 mM Mg acetate, 50 mM Na acetate, 0.01 M HEPES, pH 7.2 and proteolysis inhibitors. Fourth lane: axonemes severed by 0.035% NP-40 in the presence of 0.01 M HEPES, pH 7.2 and proteolysis inhibitors. Fifth and sixth lane: soluble proteins extracted from axonemes at the time of deflagellation by 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 the fifth and sixth lane, respectively. Bands referred to as 1α, 1β, 2', 3, and 3' are indicated at the left side.

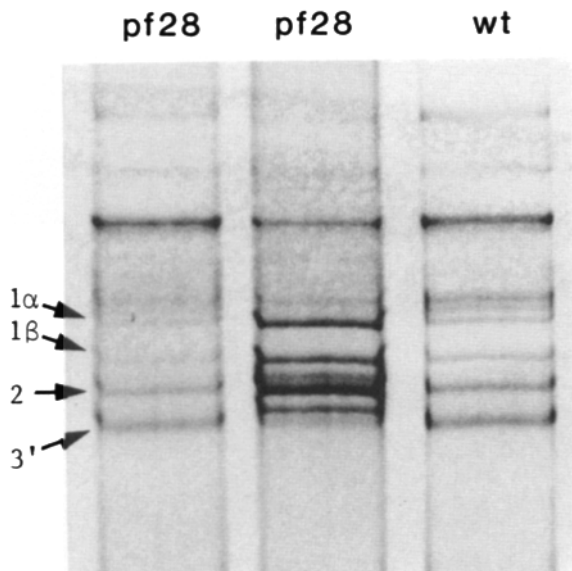


Figure 4. Electrophoretogram of ^{35}S -labeled flagellar polypeptides. First and second lane: axonemes of the mutant *pf28* severed by exposure to 0.035% NP-40 in the presence of 0.01 M HEPES, pH 7.2 and proteolysis inhibitors and by the pH shock method, respectively. Third lane: axonemes of wild-type 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 1 α , 1 β , 2, and 3' are indicated at the left side.

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 1 α , 1 β , and 2 are located in the proximal portion of flagella. On the other hand, the inner arms formed by soluble heavy chains 1 α , 1 β , 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 I2 that is distal to radial spoke S2 (19). In contrast with that interpretation, the

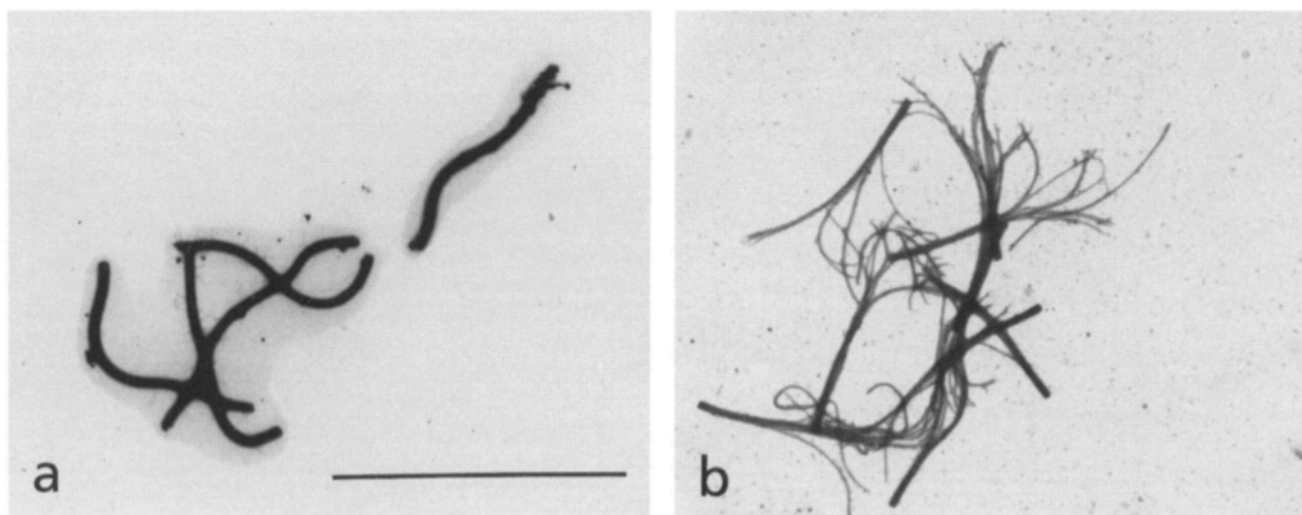


Figure 5. Electron micrographs of mutant *pf28* axonemes prepared by negative staining. (*a*) Axonemes severed by exposure to 0.035% NP-40, 5 mM Mg acetate, 0.01 M HEPES, pH 7.2. (*b*) Axonemes severed by exposure to 0.035% NP-40, 0.01 M HEPES, pH 7.2. Bar, 10 μm .

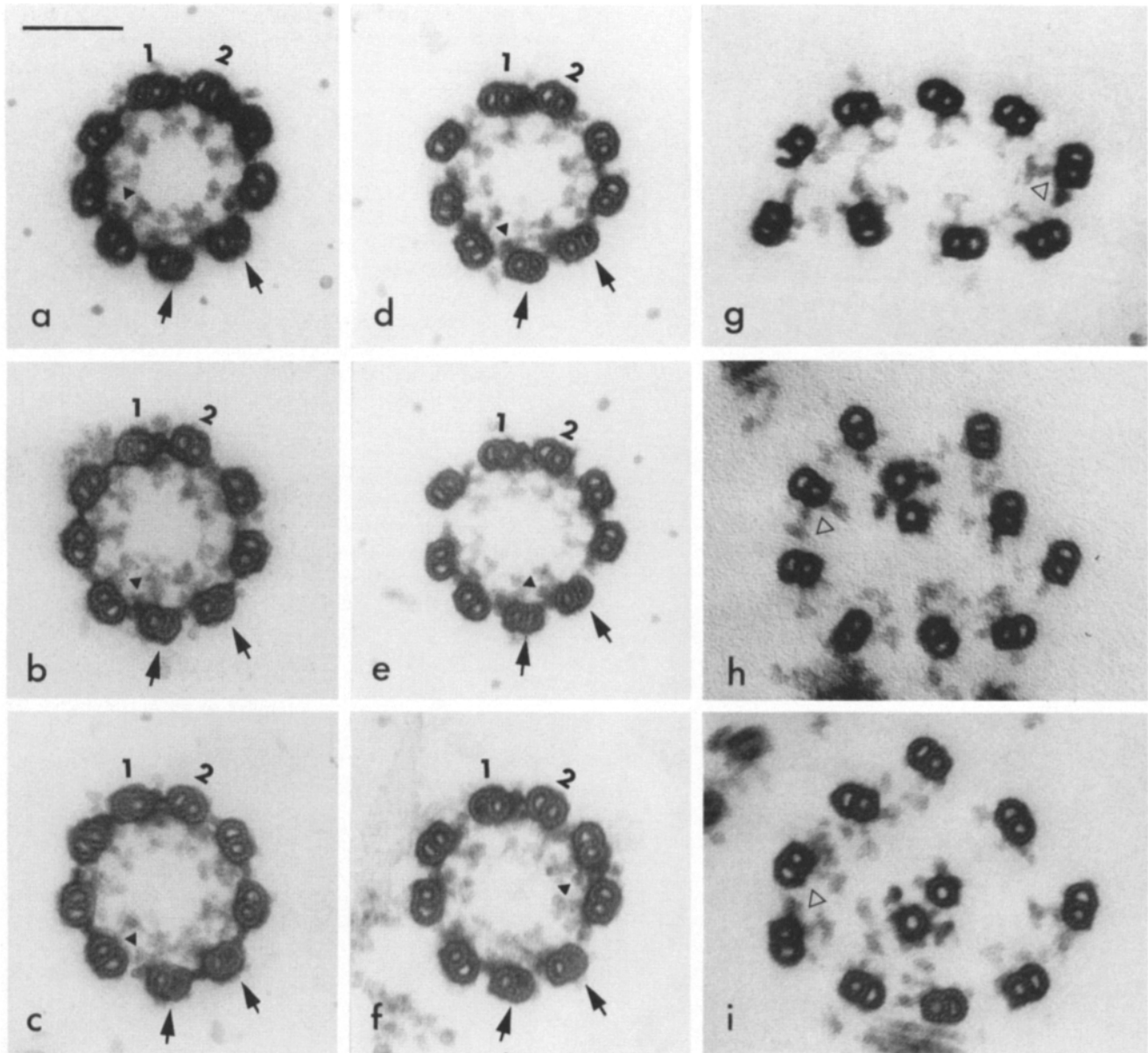


Figure 6. Thin-section electron micrographs of *pf28* axonemes severed by exposure to 0.035% NP-40, 0.01 M HEPES, pH 7.2. (a-i) Sections were ordered to show increasing extents of axonemal structure opening. (a-f) Outer doublets 1 and 2 and 5 and 6 are indicated by numbers and arrowheads, respectively. Filled triangles indicate some structures that were identified as inner arms. (g-i) Empty triangles indicate places where inner arms are missing. All sections are oriented as they are viewed from the proximal portion of the axoneme. Bar, 0.1 μm .

evidence described in preceding sections indicated that heavy chain 2' differs from heavy chain 2 for its solubility and location in the axoneme. Therefore, those heavy chains may not be part of the same structure and could instead form different inner arms.

To investigate this point further we turned to the analysis of inner arm heavy chains of the arm defective mutant *pf22* that lacks most of outer arm heavy chains and one inner arm heavy chain (9). The identification of the defective inner arm heavy chain was not achieved in previous analyses and could help to determine whether heavy chains 2' and 2 are subunits of different inner arm. Flagella of the mutant *pf22* lack heavy chain 2 entirely and are deficient for heavy chain 3 (Fig. 7).

The mutants *pf30* lacking inner arm I1 and *pf23* lacking I1 and I2 were adopted as standards and analyzed as recombinants with the mutant *pf28* to eliminate the interference of outer arm heavy chains in the electrophoretic resolution of inner arm heavy chains (19). Flagella of the strain *pf30pf28* lack heavy chains 1 α and 1 β and flagella of *pf23pf28* lack heavy chains 1 α , 1 β , 2', and 2 as reported previously (19). The strains *pf30pf28*, *pf23pf28* and *pf22* all have flagella <5 μm long (Table I) and, as expected, they are all defective also for heavy chains 3' and 2 (Fig. 7).

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-

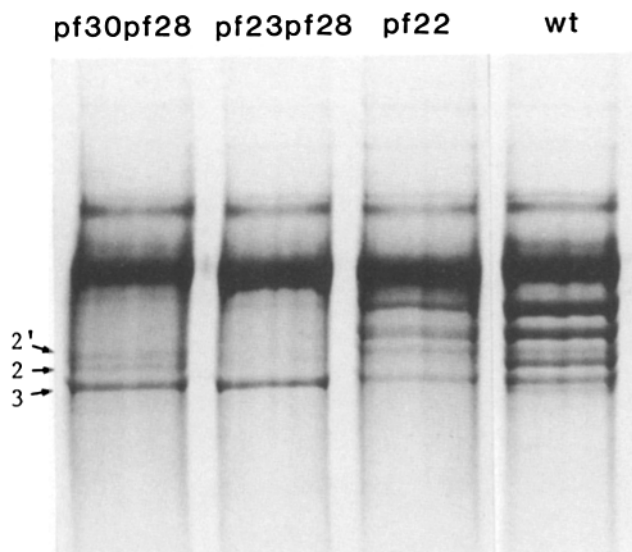


Figure 7. Electrophoretograms of ^{35}S -labeled flagellar polypeptides of the recombinant strains *pf30pf28*, *pf23,pf28*, the mutant *pf22* and wild-type cells. Equal amounts of radioactivity were analyzed in each lane. Bands referred to as 2', 2, and 3 are indicated at the left side.

tively. In addition, *pf22*, *pf23*, and *pf30*, mutations affect a different subset of inner arm heavy chains 2', 2, and 3. This evidence suggests that each heavy chain 2', 2 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 *Chlamydomonas* axonemes that includes the following features. First, the row is formed by three inner arms repeating every 96 nm. Second, each inner arm, II, I2, and I3, is composed of two distinct heavy chains

referred to as 1 α and 1 β , 2 and 2' and 3 and 3', respectively. Finally, inner arms I2 and I3 are more similar to each other than to inner arm II.

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 II and all heavy chains of inner arm I2 and I3 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 α and 1 β . 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

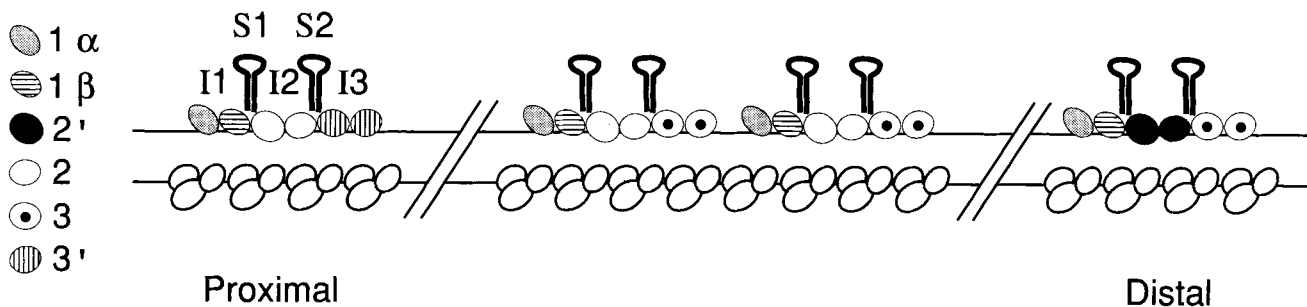


Figure 8. Schematic representation of inner arm triplets forming the inner arm row as they could be viewed in a longitudinal section of outer doublets. The proximal end of the axoneme is oriented to the left. Each inner arm heavy chain is represented by an ellipse that is differentiated as indicated at the left side. Inner arm II is composed of heavy chains 1 α and 1 β along the axoneme. Inner arm I2 is composed of two heavy chains 2 in the proximal and middle regions and of two heavy chains 2' in the distal region. Inner arm I3 is composed of two heavy chains 3' in the proximal region and of two heavy chains 3 in middle and distal regions. Radial spokes are referred to as S1 and S2 and outer dynein arms are represented as three overlapping ellipses.

be located exclusively at the distal region of the axoneme because they are present together with heavy chains 1 α and 1 β 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 axoneme. 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 μ m long.

Heavy chain 3' is present only in flagella >6 μ m 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 recombinant 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 μ m long independently from their motility. Second, proximal inner arms I2 and I3 are assembled to 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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