Striated Flagellar Roots: Isolation and Partial Characterization of a Calcium-modulated Contractile Organelle

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ABSTRACT We report the isolation of striated flagellar roots from the Prasinophycean green alga *Tetraselmis striata* using sedimentation in gradients of sucrose and flotation on gradients of colloidal silica. PAGE in the presence of 0.1% SDS demonstrates that striated flagellar roots are composed of a number of polypeptides, the most predominant one being a protein of 20,000 M_r. The 20,000 M_r protein band represents ~63% of the Coomassie Brilliant Blue staining of gels of isolated flagellar roots. Two-dimensional gel electrophoresis (isoelectric focusing and SDS PAGE) resolves the major 20,000 M_r flagellar root protein into two components of nearly identical M_r, but of differing isoelectric points (i.e., pI's of 4.9 and 4.8), which we have designated 20,000-M_r-α and 20,000-M_r-β, respectively. Densitometric scans of two-dimensional gels of cell extracts indicate that the 20,000-M_r-α and -β polypeptides vary, in their stoichiometry, between 2:1 and 1:1. This variability appears to be related to the state of contraction or extension of the striated flagellar roots at the time of cell lysis. Incubation of cells with $^{32}PO_4$ followed by analysis of cell extracts by two-dimensional gel electrophoresis and autoradiography reveals that the more acidic 20,000-M_r-β component is phosphorylated and the 20,000-M_r-α component contains no detectable label. These results suggest that the 20,000-M_r-α component is converted to the more acidic 20,000-M_r-β form by phosphorylation. Both the 20,000-M_r-α and -β flagellar root components exhibit a calcium-induced reduction in relative electrophoretic mobilities in two-dimensional alkaline urea gels. Antiserum raised in rabbits against the 20,000-M_r protein binds to both the 20,000-M_r-α and 20,000-M_r-β forms of the flagellar root protein when analyzed by electrophoretic immunoblot techniques. Indirect immunofluorescence on vegetative or interphase cells demonstrate that the antibodies bind to two cyclindrical organelles located in the anterior region of the cell. Immunocytochemical investigations at ultrastructural resolution using this antiserum and a colloidal gold-conjugated antirabbit-IgG reveals immunospecific labeling of striated flagellar roots and their extensions. We conclude that striated flagellar roots are simple ion-sensitive contractile organelles composed predominantly of a 20,000 M_r calcium-binding phosphoprotein, and that this protein is largely responsible for the motile behavior of these organelles.

Motility in eucaryotic cells in general appears to operate under calcium control through the regulatory action of a large family of calcium-modulated proteins (18, 19, 26). Calcium-based regulation of motility is best understood for troponin C and calmodulin in actomyosin contraction (sliding) in skeletal and smooth muscle, and in various nonmuscle actin-based cell movements (11, 13, 17, 47, 50). Evidence suggesting that calcium and calmodulin are involved in ciliary microtubule-based movement, i.e., sliding, (15, 22, 30) and in regulation of the mitotic apparatus (48, 49) is accumulating. All of these processes represent highly derived motility mechanisms that involve complex interactions between several distinct proteins and their regulatory components. A group of calcium-sensitive contractile organelles distinct from the actomyosin- and

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microtubule-based systems have recently come to attention (cf. references 7–9). Such organelles have been most completely characterized for the ciliates *Vorticella* and *Zoothamnium*, and have been shown to be composed primarily of a low molecular-weight protein called "spasmin" (2–4, 36). We present new evidence for the wider occurrence of spasminlike proteins in organelles called striated flagellar roots. Striated flagellar roots occur in association with the basal apparatus of many flagellated and ciliated eucaryotic cells (35, 43). Striated flagellar roots are contractile organelles (40). Functional considerations have been somewhat speculative (28, 29, 31, 39, 40), however, due to the paucity of information concerning striated flagellar root composition and responsiveness to physiological changes in free calcium. We present new observations on striated flagellar roots, which suggest that these calcium-sensitive contractile organelles are simple in composition. Flagellar roots appear to be composed, in large part, of a 20,000-M_r phosphoprotein, which undergoes a mobility shift in alkaline urea gels, characteristic of several known calcium-binding proteins.

MATERIALS AND METHODS

Cultures: *Tetraselmis striata* Butch. (No. 443) was obtained from Dr. J. C. Green, Plymouth Culture Collection, United Kingdom. Cultures were grown in filtered seawater enriched with 10% soil water extract, 200 mg/liter $NaNO_3$, and 20 mg/liter $NaHPO_4 \cdot 7H_2O$ at 18°C on a 16-h light/8-h dark cycle and constantly bubbled with air. For flagellar root isolation, 18–20 liters of cells were harvested by centrifugation, washed in 0.3 M NaCl and 0.1 mM $CaCl_2$, and stored frozen at −20°C.

Flagellar Root Isolation: Frozen cells were defrosted in the isolation buffer containing 0.1 M NaCl, 0.5 mM $MgCl_2$, 30 mM 2-[*N*-morpholino]-ethanesulfonic acid, 3 mM NaN_3, 2 mM EGTA, 0.5% Triton X-100, and 5 mM phenylmethylsulfonyl fluoride. The free calcium level was adjusted to pCa 4 by the addition of $CaCl_2$, and pH adjusted to 6.8 with KOH. Cells were washed two or three times in the isolation buffer by centrifugation at 121 *g* for 5 min to remove old mother cell walls (thecae) and other debris.

Cells were lysed as a 20% suspension (packed cell vol:isolation buffer) at 0–4°C by sonication (100 W) using a Heat Systems Sonifier model W185D (Plainview, NY) equipped with a macroprobe. Eight periods of sonication (10 s each) were applied between intermittent 30-s cooling periods. Cell brei was sedimented at 121 *g* for 5 min, and the supernatant fraction was collected and centrifuged at 12,000 *g* for 10 min. The resulting pellet was resuspended in 30 ml of isolation buffer with 12 strokes of a tight-fitting Dounce homogenizer and centrifuged again at 12,000 *g* for 10 min. This was repeated three to five times until no further chlorophyll was extracted into the supernatant fraction. The pellet was then resuspended by homogenization in 5 ml of isolation buffer, and layered onto a discontinuous sucrose gradient composed of a 10-ml cushion (2.0 M) and a 20-ml step (1.5 M). The gradient was centrifuged for 30 min at 4,000 *g* in a Sorval HB4 swinging-bucket rotor (DuPont Instruments, Sorval Biomedical Div., Newtown, CT). The 1.5 M sucrose zone was harvested, diluted with 5 vol of isolation buffer, and centrifuged at 12,000 *g* for 15 min. The pellet was resuspended by homogenization in a mixture of 1 vol of colloidal polyvinyl propylene-coated silica (Percoll, Pharmacia Fine Chemicals, Piscataway, NJ) and 1 vol of isolation buffer. This preparation was centrifuged at 27,000 *g* for 30 min in a Sorval SS34 fixed-angle rotor. Flagellar roots floated to the buffer-colloidal silica interface, and were harvested, diluted, and pelleted by centrifugation as before. Final enrichment was performed by sedimentation through a continuous sucrose gradient (1.0–2.0 M) at 50,000 *g* for 1 h in a Beckman SW27.1 swinging-bucket rotor (Beckman Instruments, Inc., Fullerton, CA). Flagellar roots sediment as a milky zone to ~1.22 g/ml.

PAGE: (*a*) SDS PAGE was carried out according to the method of Laemmli (20) in slab gels containing 0.1% SDS and either 15% or a 5–15% gradient of polyacrylamide. (*b*) Two-dimensional electrophoresis with isoelectric focusing for separation in the first dimension and SDS PAGE in the second dimension was carried out according to the O'Farrell (33) procedure. 2% total ampholines composed of 1.6% Biolyte 4-6 and 0.4% Biolyte 3-10 (Bio-Rad Laboratories, Richmond, CA) were used in the first dimension. The two-dimensional O'Farrell gels are printed with the acidic end at the right and the low-molecular-weight region of the SDS gel at the bottom. (*c*) Two-dimensional alkaline urea gels were run in the presence or absence of $CaCl_2$ according to the methods of Head and Perry (16) and Routledge et al. (36). Gels were stained by the ammonical silver nitrate method (34) or using 0.1% Coomassie Brilliant Blue in 50% methanol and 7% acetic acid, then destained in 5% methanol and 7% acetic acid. Gel slices were scanned at 580 nm with an ISCO UA-5 monitor (Lincoln, NE) and recorder equipped with a gel pusher. Peak areas were calculated by two methods: multiplication of peak height × peak width at 1/2 peak height, or by cutting out appropriate traces of the recorder plot and weighing them (6).

Antigen Preparation and Immunization: Samples of isolated flagellar roots (2–3 mg protein), solubilized in SDS PAGE sample buffer and applied as a continuous curtain were electrophoresed in 15% polyacrylamide slab gels (15 × 12 × 0.3 cm), and stained with Coomassie Brilliant Blue. The 20,000-M_r flagellar root protein was excised from the gel with a razor blade, minced, and electrophoretically eluted in 25 mM Tris-HCl, 80 mM glycine (pH 8.6), and 0.1% SDS using an ISCO protein concentrator. After extensive dialysis against PBS, this protein was used to immunize young female New Zealand rabbits. 10 subcutaneous injections containing 0.5 mg of protein (total) in complete Freund's adjuvant, and boosts (0.1 mg of protein) in incomplete adjuvant on day 14, and subsequently every 2 wk for 2 mo were used. Preimmune and immune sera were collected from the marginal ear veins. Extracts of striated flagellar roots were analyzed by immunoelectrophoresis (25) and flagellar roots and whole cell extracts were analyzed by the gel transfer and immunostaining procedure of Towbin et al. (45).

Phosphate ($^{32}PO_4$) Labeling: Cells were grown and harvested as indicated above, washed three times in an artificial seawater (ASW)[1] containing no added PO_4 (ASW-P: 300 mM NaCl, 8 mM KCl, 25 mM MgCl, and 5 mM Na_2SO_4, adjusted to pH 6.8) and resuspended in ASW-P at a cell density of ~10^7 cells/milliliter. Carrier-free $^{32}PO_4$ (New England Nuclear, Boston, MA) was added (0.1 mCi/ml, final) and the cells were incubated at 24°C for up to 1 h on a rotator. Cells were collected by centrifugation and washed three times in ASW-P. The final pellet, corresponding to ~10^6 cells was resuspended in 300 μl of isoelectric focusing lysis buffer, sonicated, and centrifuged to remove undissolved residue and theca. After electrophoresis and staining, gels were dried and autoradiographs recorded on Kodak XAR film (Eastman Kodak Co., Rochester, NY), using a Dupont Cronex Quanta III screen (DuPont Instruments, Wilmington, DE) for the times indicated in the figure legends. Immunoprecipitation of ^{32}P-labeled protein was carried out after lysis of whole cell preparations in 100 μl of 2% SDS, 0.5% deoxycholate, and 0.5% Nonidet P-40, and brief sonication. Insoluble material was pelleted by centrifugation at 15,000 *g* for 3 min, and the lysate was diluted with 9 vol of buffer containing 2% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl (pH 8.6), 2 mM EDTA. 1 μl of preimmune serum or antiserum directed against the 20,000 M_r flagellar root protein was added and allowed to react overnight at 4°C. 20 μl packed volume of Protein A-Sepharose (Pharmacia Fine Chemicals) was added and, after 1 h of incubation, washed three times and then dissolved in SDS PAGE sample buffer and analyzed by electrophoresis and radioautography.

Indirect Immunofluorescence: Cells were fixed in 3% fresh formalin made up in ASW for 30 min, washed in water, dehydrated through ethanol, cleared with xylene and infiltrated with paraffin. Sections, ~3 μm thick, were mounted on glass slides, deparaffinized with xylene, hydrated through a descending ethanol series, and brought into PBS, pH 7.2. Sections were incubated for 30 min at 37°C in primary anti-20K immune serum or preimmune serum (1:250 dilution), washed in PBS, incubated in fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA), washed and mounted in 10% glycerol in PBS. Observations were made with a Zeiss Axiomat microscope (Carl Zeiss, Inc., New York) equipped with epi-illumination for fluorescein excitation and were recorded on Kodak Tri-X film developed in Acufine developer.

Immuno-Gold Labeling: Sections were fixed using 3% glutaraldehyde made up in ASW for 30 min, washed in water, dehydrated through an ethanol and propylene oxide series, infiltrated with Epon 812 resin, and cured at 60°C. Sections with silver interference colors, collected on 200-mesh copper grids, were etched using 10% H_2O_2 for 15 min, washed in PBS and incubated at 37°C for 2 h with primary anti-20-Kdalton immune serum or preimmune serum diluted 1:50 in PBS. After washing in PBS the sections were incubated for 1 h at 37°C with 5-nm colloidal gold conjugated to goat anti-rabbit IgG made according to De Mey (12; courtesy of Dr. S. Ogihara and Dr. J. Condeelis). The material was rinsed with PBS followed by water, and poststained with uranyl acetate. Osmium tetroxide fixation and lead citrate postsection staining were not used on the immuno-gold-labeled material.

Electron Microscopy: "Calcium Shock": Living cultures were harvested, washed in ASW lacking added calcium (ASW-Ca) and incubated in this medium for 30 min. Samples were "calcium-shocked" by the addition of 2 mM $CaCl_2$ and then fixed within 30 s. Fixation was carried out for 1 h at

[1] *Abbreviation used in this paper:* ASW, artificial seawater.

room temperature by addition of an equal volume of ASW containing 4% glutaraldehyde. Samples were washed twice, postfixed in aqueous 1% OsO_4, dehydrated in an ethanol series with propylene oxide as the transition fluid, infiltrated with Epon 812 resin and cured. Thin-sections were stained with 1% uranyl acetate and lead citrate and then observed and photographed using a JEOL 100 CX electron microscope (JEOL USA, Electron Optics Div., Peabody, MA).

RESULTS

Calcium Shock-induced Flagellar Root Contraction

Tetraselmis is a quadriflagellate unicellular green alga. Each cell has two striated flagellar roots which are associated proximally with the flagellar apparatus and distally with the plasmalemma (24, 27, 37, 41). The striated flagellar roots of *Tetraselmis* are contractile organelles (40). Fig. 1 illustrates two *Tetraselmis* cells sectioned through the narrow plane of these broadly oval and flattened cells. The cell in Fig. 1 *A* was fixed in ASW-Ca in the absence of added calcium, whereas the cell in Fig. 1 *B* was fixed within 30 seconds of adding $CaCl_2$ (2 mM) to the cell suspension. Contraction of the flagellar roots (Fig. 1 *B*) results in in-pocketing of the plasmalemma distally (double-headed arrow, *S*), and proximally in deflagellation and displacement of the flagellar apparatus (*FA*). In-pocketing of the plasma membrane can be monitored at the light microscope in living cells caused to tumble across the field of view. This in-pocketing was observed to be cyclic in some cells. Cells can recover completely from calcium shock, regrowing flagella and becoming motile within several hours of being returned to culture conditions.

Fractions Enriched in Flagellar Roots

Fig. 2 illustrates a preparation enriched in flagellar roots from the final continuous sucrose gradient (see Materials and Methods). Flagellar roots were isolated in a fully contracted

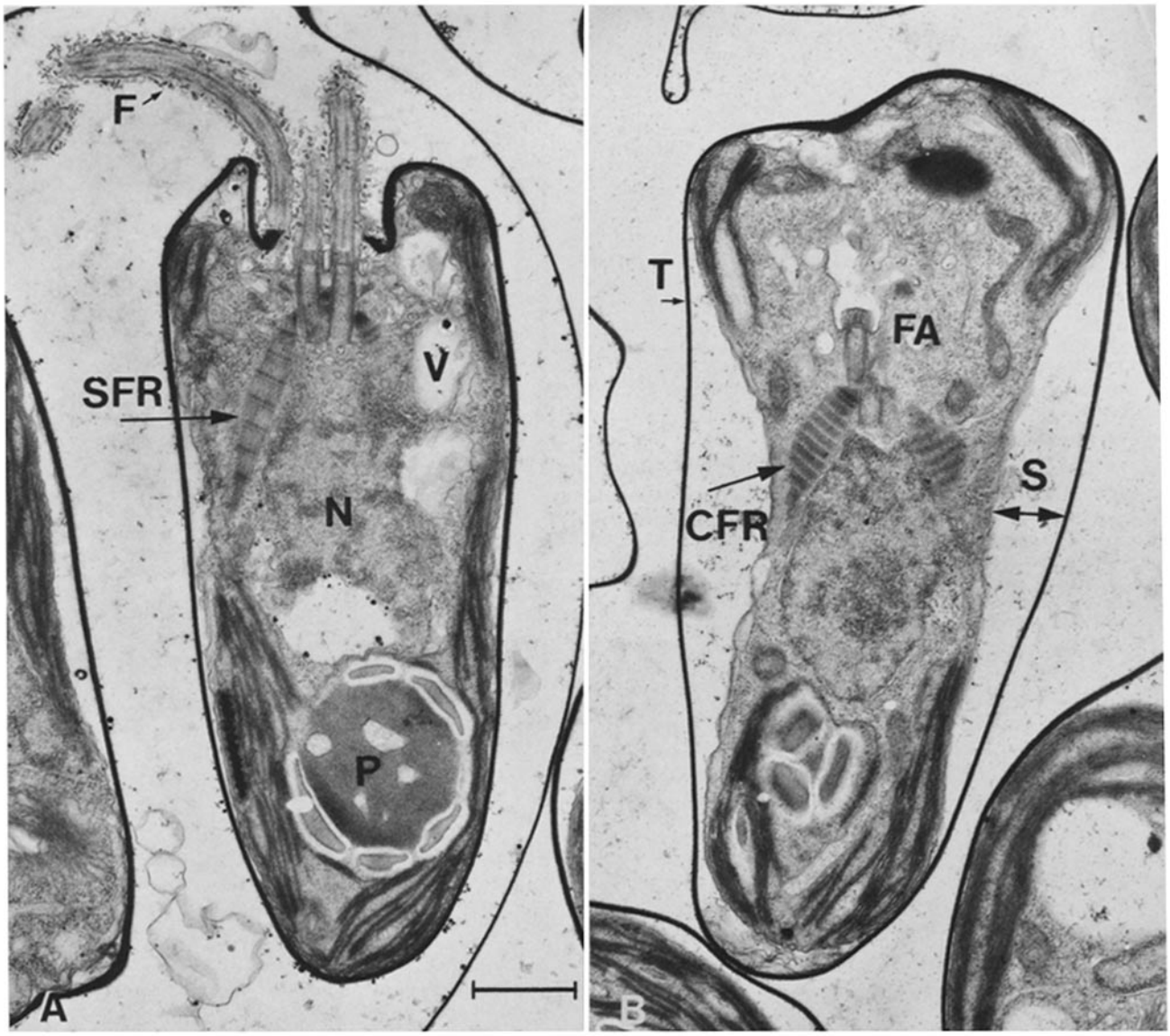


FIGURE 1 Striated flagellar roots are contractile organelles. (*A*) A thin-sectioned *Tetraselmis* cell fixed in the absence of added calcium. One of the two fully extended striated flagellar roots (*SFR*) of this cell is visible. (*B*) A thin-sectioned *Tetraselmis* cell fixed within 30 s of a "calcium shock." Both flagellar roots have contracted (*CFR*) resulting in deflagellation, displacement of the flagellar apparatus (*FA*), and in an inpocketing of the plasmalemma (*S*). *V*, vacuole; *N*, nucleus; *P*, pyrenoid; *T*, theca; *F*, flagella. Bar, 1 μm. × 15,000.

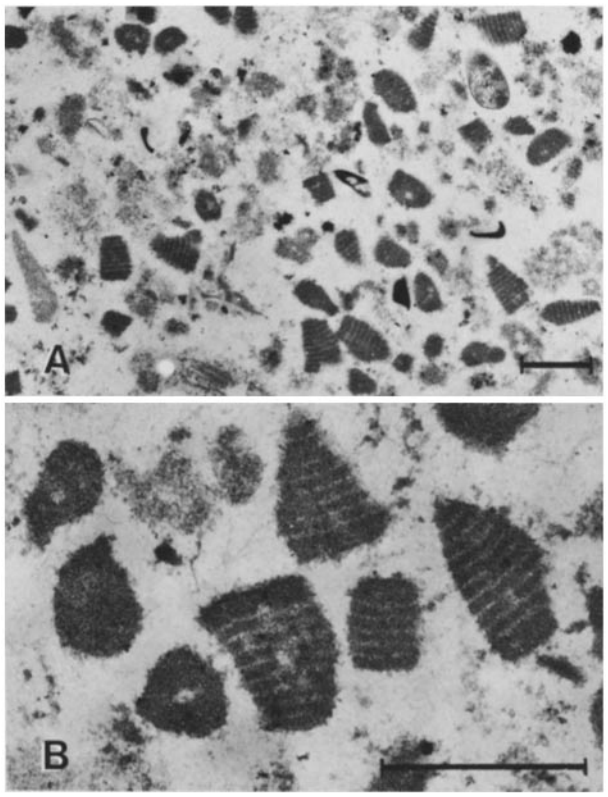


FIGURE 2 Fraction from a continuous sucrose density gradient enriched for striated flagellar roots. Striated flagellar roots isolated in buffers with pCa 4 are fully contracted. (*A*) Low-magnification view. (*B*) Higher magnification. Bar, 1 μm.

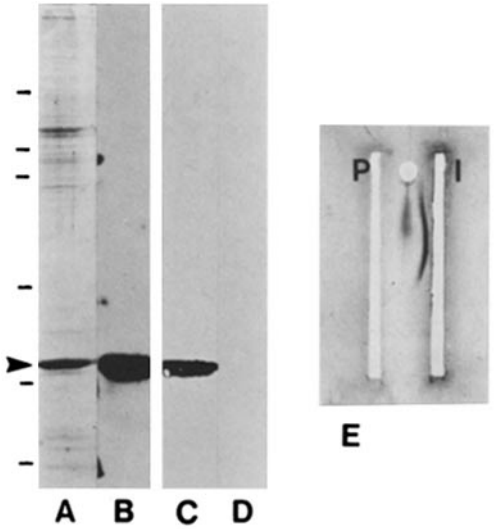


FIGURE 3 SDS PAGE analysis of *Tetraselmis* flagellar root preparations. Lane *A*, 3 μg of protein; demonstrates a principle Coomassie Brilliant Blue staining band at 20,000 M_r (arrowhead). Several minor components between 50,000 and 60,000 M_r and in the low-molecular-weight region are also evident. Preparative SDS PAGE was used to purify the 20,000-M_r protein for immunization of rabbits. Lane *B* shows a Coomassie-stained sample (3 μg) of the purified antigen. Immunoblot analysis of nitrocellulose transfers of a flagellar root gel similar to that in lane *A* shows anti-20,000 M_r specific staining (lane *C*) and no preimmune reaction (lane *D*). Molecular weight markers indicated at the left are, top to bottom: 68,000, 45,000, 34,700, 24,000, 18,400, and 14,300 M_r. Immunoelectrophoresis (*E*) run against urea extracts of flagellar roots, demonstrate anti-20,000-M_r immune specific immunoprecipitation resulting in a single arc (trough *I*) and no preimmune precipitation (trough *P*).

state in an isolation buffer of pCa 4. Clearly, flagellar roots are the predominant organelle in this fraction (Fig. 2). The principal identifiable contaminant appears to be short segments of thecae (cell wall). Isolated flagellar roots have a triangular appearance, measure ~0.75 μm when sectioned longitudinally, and consist of 10–12 densely staining amorphous zones. Cross-sectioned roots have various shapes; often they appear as triangular structures that display an amorphous substructure.

Flagellar Root Composition and Anti-20,000-M_r Serum Characterization

Analysis of isolated flagellar roots by SDS PAGE indicates that the predominant Coomassie Brilliant Blue staining band has a relative mobility corresponding to an apparent molecular weight of 20,000 (Fig. 3*A*). The 20,000-M_r protein represents ~63% of the Coomassie Brilliant Blue staining material in gels of the isolated striated flagellar roots. A number of other components are present at low levels in flagellar root preparations, including several between 50,000 and 60,000 M_r, and several in the lower molecular-weight range.

The 20,000 M_r protein was purified by preparative SDS PAGE (Fig. 3*B*) and used to elicit antibodies in rabbits. Fig. 3*C* illustrates immune serum staining of only the 20,000 M_r band of a flagellar root SDS polyacrylamide gel lane (similar to Fig. 3*A*) after transfer to a nitrocellulose sheet, whereas preimmune serum (Fig. 3*D*) shows no detectable reaction. Immunoelectrophoretic analysis demonstrates immune specific (Fig. 3*E*, trough *I*) precipitation resulting in a single arc when tested against flagellar root extracts.

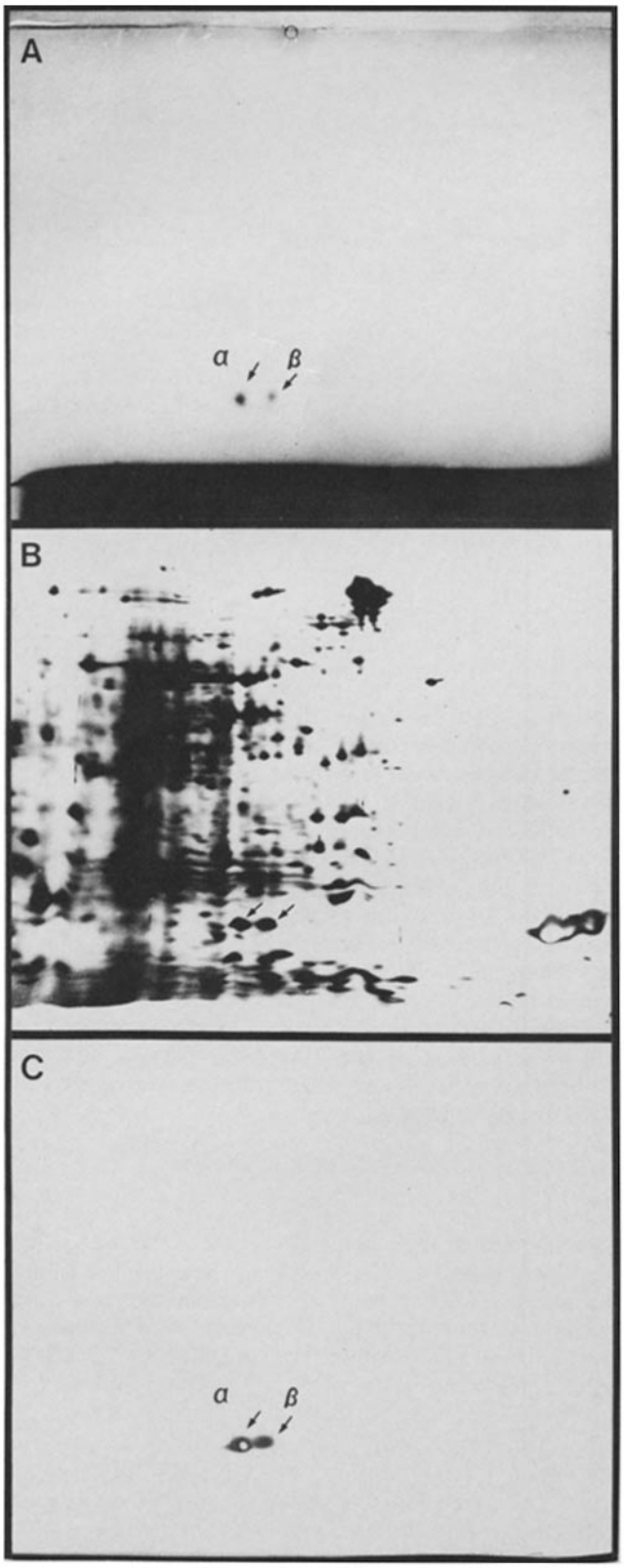
Analysis of 6 M urea extracts of flagellar root preparations by two-dimensional PAGE utilizing isoelectric focusing in the first dimension and SDS PAGE in the second dimension, demonstrates that the 20,000 M_r root protein is composed of two acidic components, which we call 20,000-M_r-α and 20,000-M_r-β (Fig. 4*A*). These components have estimated isoelectric points of 4.9 and 4.8, for α and β, respectively. Both 20,000-M_r-α and -β polypeptides can be identified in two-dimensional O'Farrell gels of whole cell preparations on the basis of their migration (Fig. 4*B*) and by immunoblot analysis of whole cell two-dimensional gel transfers using antisera specific for the 20,000-M_r flagellar root protein (Fig. 4*C*). Both 20,000-M_r-α and 20,000-M_r-β flagellar root components react with immune serum, whereas no other proteins show detectable reaction.

Variable Stoichiometry of 20,000-M_r-α and 20,000-M_r-β

Comparison of the relative amounts of 20,000-M_r-α and -β by densitometric scans of Coomassie Brilliant Blue-stained gels (Fig. 5) illustrates a variable stoichiometry of α to β, which ranges from 2:1 to 1:1. Changes in the ratio of α to β relate to the state of contraction or extension of striated flagellar roots immediately before cell lysis. Preparations from calcium-shocked cells, where most of the flagellar roots are contracted, show a higher ratio of α to β (Fig. 5, trace *a*). Preparations from cells that were not calcium shocked contain a mixture of extended, and partially contracted striated flagellar roots and these show a lower α to β ratio (Fig. 5, trace *b*).

Protein Phosphorylation

Tetraselmis cells rapidly incorporate $^{32}PO_4$ label into a number of proteins. Two dimensional O'Farrell gel analysis and radioautography demonstrates that the 20,000-M_r-β component of striated flagellar roots is a major acidic phosphoprotein of the cell (Fig. 6, *B* and *C*), whereas the 20,000-M_r-α component shows no detectable incorporation of label.



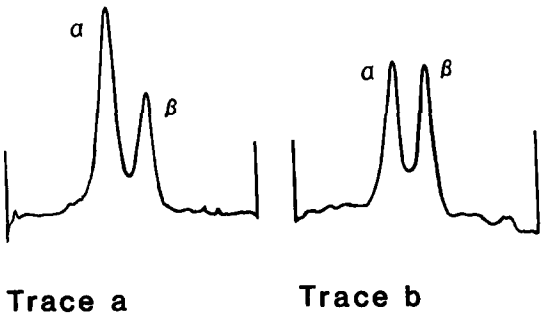


FIGURE 5 The ratio of α to β in cell extracts is variable. Tracings of densitometric scans of regions of two-dimensional gels of cell extracts in the region of the α and β spots. Trace *a* is from preparations in which the cells were calcium shocked before cell lysis, the ratio of α to β is 2:1. Trace *b* is from preparations of cells that were not calcium shocked before lysis: here the ratio of α to β is nearly 1:1.

Prolonged exposure of the radioautogram (Fig. 6*C*) reveals many phosphoproteins in addition to the 20,000-M_r-β component. In addition, the anti-20,000-M_r serum specifically immunoprecipitates a ^{32}P-labeled 20,000-M_r protein from *Tetraselmis* whole cell extracts (Fig. 6*D*). Thus, the variable stoichiometry of α to β Coomassie staining bands probably corresponds to conversion of 20,000-M_r-α into 20,000-M_r-β by phosphorylation.

Alkaline Urea Gel Electrophoresis

Both the α and β components undergo a reduction in mobility when electrophoresed in polyacrylamide gels containing 6 M urea and millimolar free calcium (Fig. 7). This mobility shift was best demonstrated in two-dimensional gels where the protein was electrophoresed in low free calcium levels in the first dimension and either low or high free calcium levels in the second dimension. Under these conditions the 20,000-M_r-α and -β components undergo a 16 and 24% reduction, respectively, in relative mobility in the presence of excess calcium.

Indirect Immunofluorescence and Immuno-Gold Labeling

Because the theca or cell wall of *Tetraselmis* posed a barrier to antibody penetration, both immunofluorescence and immunoelectron microscopic investigations were carried out on sectioned material. Analysis of fluorescent images (Fig. 8*B*) of sections treated with the anti-20,000-M_r immune serum reveals two cylindrical structures, often forming a V shape,

FIGURE 4 (*A*) Analysis of a 6 M urea extract of flagellar roots, 1 μg of protein by two-dimensional gel electrophoresis with isoelectric focusing in the first dimension (IEF) and SDS PAGE in the second dimension reveals two Coomassie Brilliant Blue staining polypeptides of 20,000 M_r that differ in net charge (α, pI = 4.9 and β, pI = 4.8). (*B*) Analysis of cell lysates, 7 μg of protein. Silver staining reveals the 20,000-M_r-α and -β striated flagellar root components (arrows) as major acidic proteins of the organism. (*C*) Immunoblot analysis of a nitrocellulose transfer of a companion two-dimensional gel of whole cell extract demonstrates that the immune anti-20,000-M_r serum identifies both the 20,000-M_r-α and 20,000-M_r-β flagellar root components and no other proteins. The two-dimensional gels are printed with the low-molecular-weight region at the bottom and the acidic region at the right.

located in the anterior (flagellated) region of vegetative or interphase cells. Not all cells in a given section stain in this manner due to the limited probability of a section including the immunoreactive structures in an appropriate orientation. The lower portion of Fig. 8 illustrates several examples at higher magnification of selected cells stained with the anti-20,000-M_r immune serum. Fig. 8, *G–H* illustrate cells that had been subjected to a calcium shock before fixation. Preimmune controls (Fig. 8*A*) show only a low level fluorescence. A significant portion of the fluorescence in preimmune-treated samples can be attributed to residual pigment remaining in the cells, in that similar levels of fluorescence are observed in cells that have not been exposed to the secondary fluorescein conjugate (not shown).

Immunoelectron microscopy of anti-20,000-M_r-treated thin sections confirm that striated flagellar roots are the reactive structure because they label with the secondary gold conjugate (Fig. 9). Clearly, both extended (Fig. 9*A*) and contracted (Fig. 9*B*) flagellar roots show dense labeling, whereas preimmune treated controls (Fig. 9*C*) show only a sparse gold deposit.

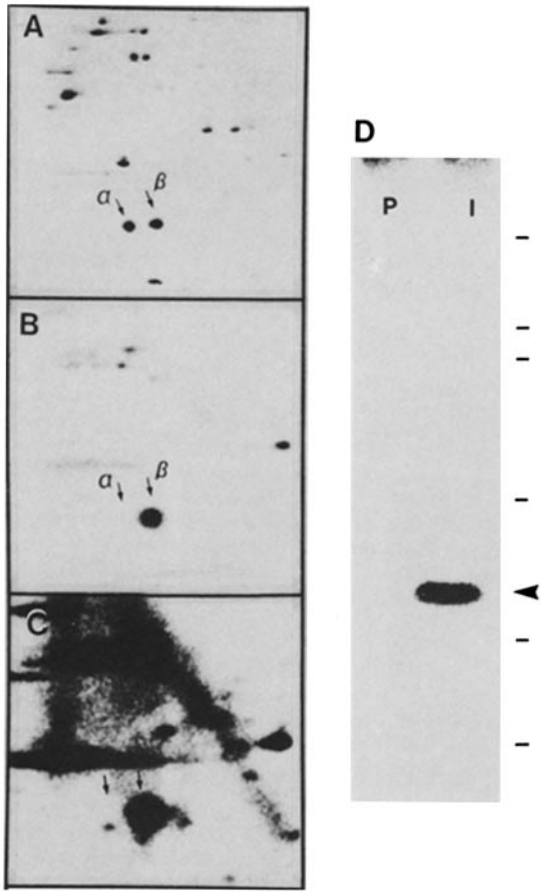


FIGURE 6 Analysis of phosphorylated proteins. Cells were incubated in $^{32}PO_4$ for 1 h before lysis. Two-dimensional gel of cell extracts, 12 μg of protein, (*A*) stained with Coomassie Brilliant Blue show the 20,000-M_r-α and -β flagellar root proteins (arrows). (*B*) Autoradiograph of the same gel shown in (*A*) after 1-h exposure illustrates that the 20,000-M_r-β flagellar root protein is a major acidic phosphoprotein, no ^{32}P-label is associated with the 20,000-M_r-α component. (C) 2-h exposure of the gel illustrates other phosphoproteins labeled under the conditions used. (*D*) Immunoprecipitation of ^{32}P-labeled flagellar root proteins. Cell lysates were treated with preimmune (*P*) or anti-20,000-M_r immune serum and precipitated as described in Materials and Methods. Clearly, the immune serum (*I*) precipitates a ^{32}P-labeled protein of 20,000 M_r and no other ^{32}P-labeled bands. Molecular weight markers indicated at right are listed in Fig. 3.

DISCUSSION

Striated flagellar roots are nearly ubiquitous in occurrence among ciliated and flagellated eucaryotic cells. Their structure varies from the "massive" roots seen in green algae such as *Tetraselmis*, in ciliated epithelia (14) and in retinal rod cells (42), to the less prominent roots associated with the primary cilia of quiescent Balb/c 3T3 fibroblasts (46). Though the fine structure of striated flagellar roots was described early in the development of biological electron microscopy (see references 14 and 23) little was known of their function, and until now, of their composition.

Here we demonstrate that preparations highly enriched for striated flagellar roots are composed predominantly of an acidic protein of 20,000 M_r. This protein has been resolved into two components by isoelectric focusing and by two-dimensional electrophoresis in alkaline urea gels run in either low or high free calcium. These two components have been designated α and β. We were interested in the physical basis for two polypeptides with nearly identical M_r's but different molecular charges. We suspected that they were similar polypeptides differing in some form of posttranslational modification. Our analysis of variable ratios of α to β in cell extracts and our demonstration that the more acidic 20,000-M_r-β component is phosphorylated leads us to suggest that the 20,000-M_r-β arises through a phosphorylation of the 20,000-M_r-α component during flagellar root extension. At this time we do not know details concerning the mechanism of 20,000-M_r-β phosphorylation; these are the subject of current investigation. We have published, in a preliminary report (39),

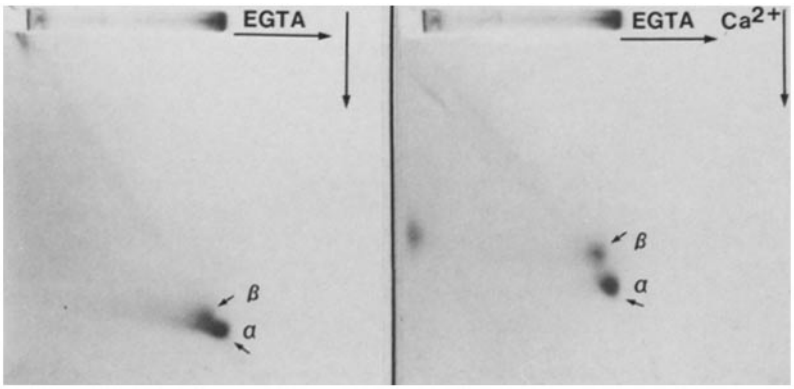


FIGURE 7 Two-dimensional alkaline urea gels of 6 M urea extracts of striated flagellar roots, 2 μg protein, run either in the presence of low or high free calcium reveal a retardation in mobility of both α and β polypeptides in the presence of excess free calcium. The samples shown here were run in the same first dimension gel in the presence of EGTA, individual lanes excised and equilibrated in either 2 mM EGTA (*left*) or 2 mM calcium (*right*) and run simultaneously in the second dimension in the presence of 2 mM EGTA (*left*) or 2 mM calcium (*right*). A separate lane from the first dimension gel has been placed above each slab to illustrate first dimension migration.

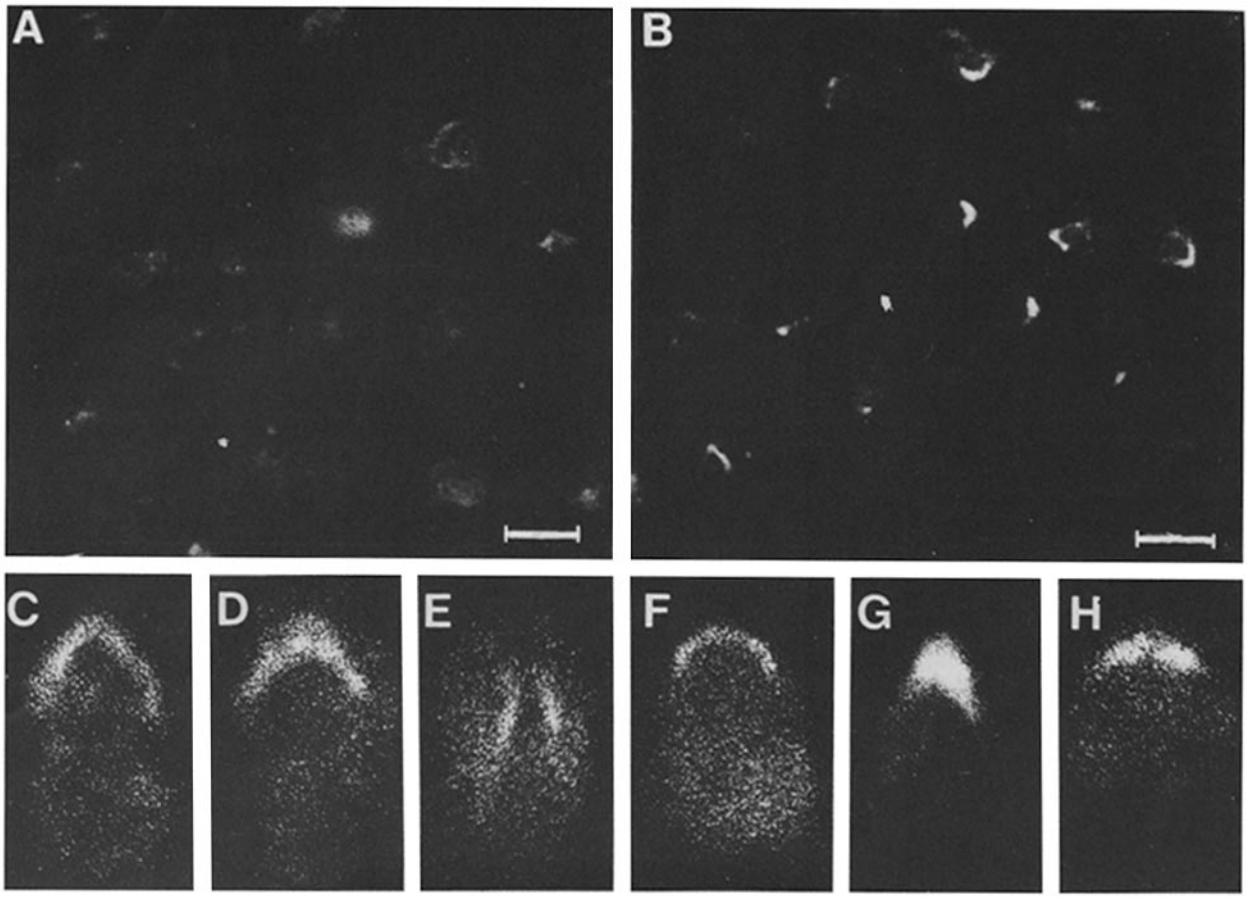


FIGURE 8 Indirect immunofluorescent staining of paraffin infiltrated and sectioned cells. (*A*) Preimmune level of background staining; (*B*) anti-20,000-M_r immune serum staining illustrating immuno-reactive V-shaped structures located in the anterior regions of appropriately sectioned cells; (*C–H*) collage of selected cells shown at higher magnification. Cells in *G–H* were subjected to a "calcium shock" prior to fixation.

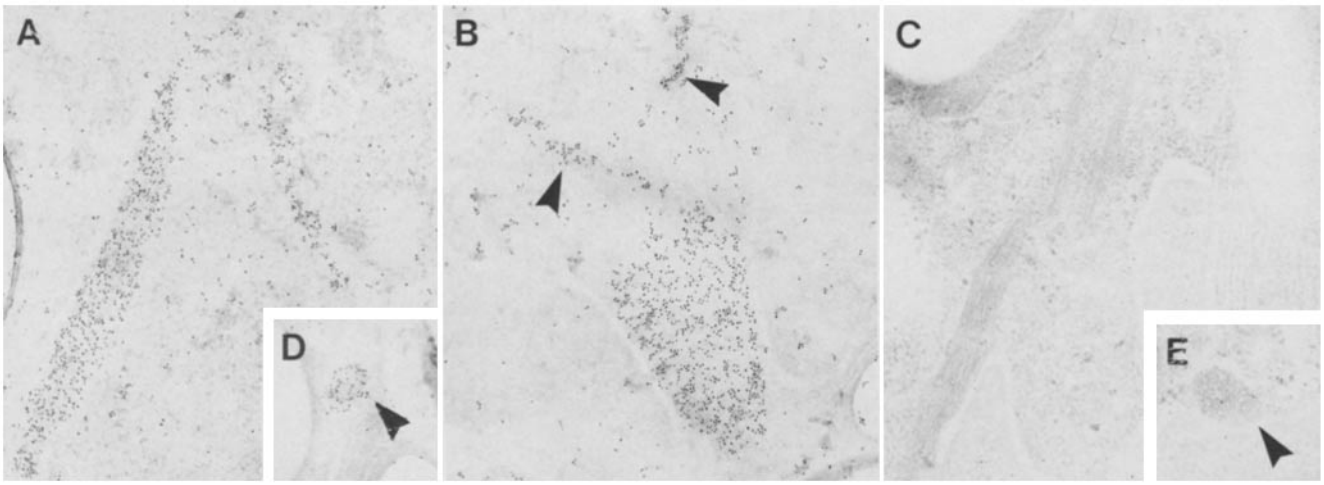


FIGURE 9 Immuno-gold labeling of glutaraldehyde fixed, epon infiltrated, sectioned cells. Anti-20,000-M_r immune serum stained sections (*A* and *B*) illustrate labeling of extended flagellar roots (*A*) their cross sections (*inset* [*D*]) and of contracted flagellar roots (*B*). While control levels of preimmune staining show only sparse gold deposits (*C* and *inset* [*E*]). × 40,000.

ATPase cytochemistry demonstrating the localization of activity to the cross striations of the flagellar roots, and Anderson (5) has reported ATPase activity associated with isolated basal bodies and associated structures from oviduct. Perhaps these activities are related to the phosphorylation and dephosphorylation of striated flagellar root proteins. We must, therefore, now consider two energy-requiring steps in striated flagellar root action. One involved in regulation of cytoplasmic free calcium levels (38) and a second more directly involved in 20,000-M_r protein phosphorylation probably during flagellar root extension.

Alkaline urea gels run in the presence of elevated calcium

illustrate that both polypeptides undergo a reduction in relative mobility suggesting direct binding of calcium to the protein. Such binding may alter the net charge and molecular conformation of the protein. In preliminary studies, we have assembled anastomosing networks of 3-nm diameter filaments from urea extracts of flagellar roots. These filaments convert into clusters of 16-nm diameter "globettes" at raised free calcium levels. These in vitro changes in morphology of reassembled filaments appear to correspond to changes in flagellar root filament morphology observed in intact organelles during contraction (cf. reference 39, Fig. 5). Our earlier observations (39) revealed distinct stages of filament shortening in situ that appeared as a twisting and supercoiling of individual filaments. The immunofluorescent and immunogold labeling studies confirm that the 20,000 M_r protein is a major structural component of striated flagellar roots. Based on the observations presented above, we suggest that the 20,000 M_r protein is the principal functional component of striated flagellar roots.

We do not know what relationship the polypeptides between 50,000 and 60,000 M_r or the other proteins, present in low amounts in our preparations, have to the flagellar roots. These proteins may be components of basal bodies or other fibrous material associated with the isolated flagellar roots. Alternatively, certain of these proteins may be accessory molecules related to striated flagellar root organization or possibly to phosphorylation.

Flagellar Roots and Spasmonemes

Fibrous flagellar roots are of at least two morphologically distinct types: (*a*) microtubule-associated striated fibers with pattern repeat of 25–35 nm (also known as System I fibers, cf. reference 27), and (*b*) striated flagellar roots, such as those studied here, composed of a bundle of 3–7-nm diameter filaments with variable striation patterns of repeat <50 nm (also known as System II fibers, cf. reference 27). System I flagellar roots of the amoeboid flagellate *Naegleria guberi* appear to be composed of a distinct class of high-molecular-weight proteins of 170, 000 M_r (21). In contrast, another study (44) on the composition of flagellar roots of the System-II type isolated from gill cells of the bay scallop *Aequipecten* suggest that these organelles are composed of a high molecular weight doublet of proteins, which Stephens (44) named "ankyrin." Analysis of the published gels, however, also reveals a major polypeptide component in the lower molecular weight region that may correspond to the 20,000 M_r protein. Further experimental analyses of striated flagellar roots from *Aequipecten* will be necessary in order to clarify the composition of these organelles.

Amos and coworkers (2–4, 36) have shown that the contractile fiber within the stalk of the ciliates *Vorticella*, *Zoothamniun*, and *Carchesium* is primarily composed of a calcium-binding contractile protein of 20,000 M_r that they call "spasmin." A preliminary investigation (Salisbury, J. L., and A. Baron, unpublished observation) demonstrates immunological cross-reactivity between the anti-20K immune serum and a 20,000-M_r component of *Vorticella* whole cell extracts by immunoblot analysis. On the basis of our results, we would suggest that striated flagellar roots of the type we have studied and the vorticellid spasmoneme are homologous organelles, and consequently that the 20,000 M_r flagellar root protein is a spasminlike molecule. If this hypothesis is correct, spasminlike contractile machines may have developed before the ancient evolutionary divergence of these two groups of organisms.

Calcium-modulated Proteins

The major 20,000 M_r striated flagellar root protein shares a number of features in common with members of the calcium-modulated regulatory protein family that includes calmodulin, parvalbumin, and troponin C (10, 18, 19). Common features of these proteins include low molecular weight (generally below 20,000), acidic isoelectric point, binding of calcium with high affinity (usually around pK_d 5–6) even in the presence of mild denaturants, and calcium-induced alterations in molecular conformation. In addition, a number of these proteins have phosphorylated forms, including calmodulin (32), myosin light chain (1) and the flagellar root protein (this study). Currently, there are no primary sequence data available for the flagellar root protein or other spasminlike proteins. Consequently, we do not know if these ion-sensitive contractile proteins share sequence homology within their calcium-binding domain(s), or with the calcium-binding domains of the calcium modulated regulatory proteins mentioned above (19). This is of particular interest in view of the fundamental role of calcium modulated regulatory proteins in motility phenomena. We postulate that ion-sensitive contractile systems such as the flagellar root represent a "primitive" motility mechanism possessing the qualities of simplicity of composition, and direct mediation of contraction by calcium binding. Perhaps, as more complex motility mechanisms arose (i.e., actomyosin- and microtubule-based systems) an evolutionary schism or divergence occurred, in which aspects of the primitive system were retained for either regulation (i.e., the calmodulin–troponin C group) or contractile (i.e., the spasminlike proteins) function.

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