Basal Body Reorientation Mediated by a Ca^{2+}-modulated Contractile Protein

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Abstract. A rapid, Ca^{2+}-dependent change in the angle between basal bodies (up to 180°) is associated with light-induced reversal of swimming direction (the "photophobic" response) in a number of flagellated green algae. In isolated, detergent-extracted, reactivated flagellar apparatus complexes of *Spermatozopsis similis*, axonemal beat form conversion to the symmetrical/undulating flagellar pattern and basal body reorientation (from the antiparallel to the parallel configuration) are simultaneously induced at $\geqslant 10^{-7}$ M Ca^{2+}. Basal body reorientation, however, is independent of flagellar beating since it is induced at $\geqslant 10^{-7}$ M Ca^{2+} when flagellar beating is inhibited (i.e., in the presence of 1 μM orthovanadate in reactivation solutions; in the absence of ATP or dithiothreitol in isolation and reactivation solutions), or when axonemes are mechanically removed from flagellar apparatuses. Although frequent axonemal beat form reversals were induced by varying the Ca^{2+} concentration, antiparallel basal body configuration could not be restored in isolated flagellar apparatuses. Observations of the photophobic response in vivo indicate that even though the flagella resume the asymmetric, breaststroke beat form 1–2 s after photostimulation, antiparallel basal body configuration is not restored until a few minutes later.

Using an antibody generated against the 20-kD Ca^{2+}-modulated contractile protein of striated flagellar roots of *Tetraselmis striata* (Salisbury, J. L., A. Baron, B. Surek, and M. Melkonian, 1984, *J. Cell Biol.*, 99:962–970), we have found the distal connecting fiber of *Spermatozopsis similis* to be immunoreactive by indirect immunofluorescence and immunogold electron microscopy. Electrophoretic and immunoblot analysis indicates that the antigen of *S. similis* flagellar apparatuses consists, like the *Tetraselmis* protein, of two acidic isoforms of 20 kD. We conclude that the distal basal body connecting fiber is a contractile organelle and reorients basal bodies during the photophobic response in certain flagellated green algae.

The recent demonstration that a variety of fibrillar structures associated with eukaryotic centrioles/basal bodies are antigenic homologs to a Ca^{2+}-modulated contractile protein has provoked much speculation concerning the functional significance of these structures (Salisbury et al., 1986; Wright et al., 1985). So called flagellar roots, basal feet, pericentriolar satellites, pericentriolar fibrils, and nucleus–basal body connectors from algal, protozoan and mammalian cell types have all been shown to be immunoreactive (Salisbury et al., 1984, 1986; Wright et al., 1985) with an antibody generated against a 20-kD protein that is the major polypeptide of the striated flagellar roots of the green flagellate *Tetraselmis striata* (Salisbury et al., 1984). However, despite the fact that the 20-kD protein has now been localized in various basal body–associated structures in organisms from diverse evolutionary lineages, there is still no established physiological function for Ca^{2+}-induced contractility of these structures.

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A number of possible functions for contractile basal body–associated fibers have been proposed: accurate basal body location within the cell (Wright et al., 1985), accurate segregation of parental and daughter basal bodies at cell division (Wright et al., 1985), redetermination of cell polarity through a Ca^{2+}-sensitive alteration of centrosomal microtubule organizing center orientation (Salisbury et al., 1986), initiation and/or directional control of flagellar beat (Salisbury and Floyd, 1978; Salisbury et al., 1981; White and Brown, 1981), regulation of gametic fusion (Melkonian, 1980*a*; Hoffman, 1973), and transient changes in relative basal body orientation (Melkonian and Preisig, 1984; Bessen et al., 1980; Watson, 1975; Melkonian, 1978; Marano et al., 1985; White and Brown, 1981).

We decided to investigate the latter phenomenon, in which it has been shown that the two basal bodies of some flagellated green algae assume different relative orientations during forward and reverse swimming, respectively (Watson, 1975; Melkonian, 1978; Melkonian and Preisig, 1984). In these algae the basal bodies are oriented antiparallel (i.e., at a relative angle of 180°) during forward swimming but un-

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dergo a reorientation through an angle of 90° each so that they can lie parallel during reverse swimming (Watson, 1975; Melkonian, 1978; Melkonian and Preisig, 1984). Ultrastructural comparison of cells, fixed during either forward or backward swimming, showed that a fibrous structure interconnecting the two basal bodies (the distal connecting fiber; Ringo, 1967) appeared more condensed in the latter, suggesting that the distal connecting fiber could be involved in basal body reorientation (Watson, 1975; Melkonian, 1978; Melkonian and Preisig, 1984).

In flagellated green algae reverse swimming occurs most often as a photophobic (escape) response, elicited by a sudden change in the photon fluence rate (Schmidt and Eckert, 1976). Photostimulation induces a transient reversal of the swimming direction (ca. 1 s) after which the organism resumes forward swimming, usually in a different direction than the original course (Ringo, 1967). Reverse thrust is created by the conversion of the flagellar wave form from the asymmetric, ciliary type beat (breaststroke) during forward swimming to the symmetrical, undulatory, flagellar type beat during reverse swimming (Ringo, 1967). Beat alteration is induced by an influx of Ca^{2+} through the surface membrane after a "step up" of the fluence rate (Schmidt and Eckert, 1976). In isolated, detergent-extracted flagellar apparatuses of *Chlamydomonas reinhardtii*, reversal of axonemal beat form occurs at 10^{-6} M Ca^{2+} (Hyams and Borisy, 1978).

Since preliminary experiments in our laboratory indicated that basal body reorientation during the photophobic response was dependent on the presence of Ca^{2+} in the medium (Schulze et al., 1986), it seemed possible that the distal connecting fiber is a Ca^{2+}-modulated contractile organelle. In this study we have used an antibody raised against the Ca^{2+}-modulated contractile protein of *T. striata* (Salisbury et al., 1984) to localize an antigenic homolog to this protein in the distal connecting fiber of a green flagellate. Contraction of the distal connecting fiber in *Spermatozopsis similis* is shown to reorient basal bodies in detergent-extracted, reactivated, isolated flagellar apparatus complexes (flagellar apparatuses)[1] at about the same physiological Ca^{2+} concentration that induces alteration of axonemal beat form. We conclude that two different motility systems are operative in light-induced reversal of swimming direction in *Spermatozopsis*.

Materials and Methods

Strains and Culture Conditions

A culture of *Spermatozopsis similis* Preisig et Melkonian (SAG 1.85) was obtained from the "Sammlung von Algenkulturen" (SAG), Pflanzenphysiologisches Institut (Göttingen, Federal Republic of Germany), and was maintained as described by McFadden and Melkonian (1986).

Light Shock Experiment

Cells of *Spermatozopsis similis* were harvested by centrifugation at 500 *g* in a benchtop centrifuge for 5 min, washed twice in one of the Ca^{2+}-buffered solutions of Bessen et al. (1980), and resuspended to a final cell density of 5×10^6 cells ml^{-1}. Cells were photostimulated by the step-up procedure of Schmidt and Eckert (1976) as modified by Hoops et al. (1984). 30 forward swimming cells in each solution were tracked, photostimulated, and their responses observed by phase contrast light microscopy.

To monitor basal body orientation before, during, and after the photophobic response, several parallel samples in either 10^{-4} or 10^{-9} M Ca^{2+} were photostimulated, then fixed (see below) at intervals thereafter. Under red illumination (690 nm), 20-µl aliquots of cell suspension were dispensed onto glass slides and left to equilibrate for 5 min. A control sample was then fixed by addition of 20 µl of 10 mM EGTA followed immediately by 20 µl of Lugol's solution (Quader and Glas, 1984). The remaining slides were then illuminated with a 1,250 W quartz halogen floodlight (Hedler, D-6257 Runkel-Lahn, FRG) from a distance of 10 cm for 2 s and fixations prepared as above at subsequent intervals.

Basal body orientation was monitored by light microscopy and verified by whole-mount EM (Gantt, 1980). The initial addition of EGTA was found to prevent basal body reorientation induced by the fixation process and had no effect on contracted connecting fibers (see Results).

Preparation of Flagellar Apparatuses

Cells were sedimented as above and then resuspended in ice-cold MT buffer (30 mM Hepes, 5 mM $MgSO_4$, 5 mM EGTA, 25 mM KCl, pH 7). After 5 min an equal volume of 2% NP-40 (Sigma Chemical Co., St. Louis, MO) in ice-cold MT buffer was added, and the lysate centrifuged in 1.5-ml microtubes in a Biofuge B (Heraeus Christ GmbH, D-3360 Osterode, FRG) at 1,000 *g* for 5 min. The green supernatant was discarded and the upper, milky portion containing the flagellar apparatuses was removed from the biphasic pellet and resuspended in 1 ml of MT buffer by vortex mixing. The lower portion of the initial pellet contained starch grains and cell debris and was discarded. The flagellar apparatuses were centrifuged again (Biofuge B, 1,000 *g* for 5 min) and resuspended in ice-cold MT buffer.

Reactivation of Flagellar Apparatuses

Flagellar apparatuses of *Spermatozopsis similis* were prepared as previously described in MT buffer (plus 1% polyethylene glycol [M_r 20,000] and 1 mM dithiothreitol [DTT]). Reactivation of flagellar apparatuses in vitro was performed as previously described (Hyams and Borisy, 1978; Bessen et al., 1980; Kamiya and Witman 1984) by suspending a 5-µl aliquot of flagellar apparatuses in 2 ml of the Ca^{2+}-buffered, ATP-containing reactivation solutions of Bessen et al. (1980). Reactivated flagellar apparatuses were observed with phase contrast optics in the perfusion chamber described by Hyams and Borisy (1978) using glass slides treated with Sigmacote (Sigma Chemical Co.).

To exclude possible effects of flagellar beating on basal body reorientation, orthovanadate (Aldrich Chemical Co., D-7924 Steinheim, FRG) was added to reactivation solutions at a final concentration of 1 µM. Alternatively, DTT and ATP were omitted from the isolation and reactivation solutions. In other experiments, flagellar apparatuses of *Spermatozopsis similis* in MT buffer were treated in a 1-ml tissue homogenizer (20 strokes; Kontes Glass Co., Vineland, NJ) to amputate the axonemes. The remaining basal body pair and connecting fiber units were pelleted (3,500 *g* for 8 min, Biofuge B [Heraeus Christ GmbH]) and aliquots then resuspended in the various Ca^{2+}-buffered solutions lacking ATP and DTT. These basal body pairs were examined for reorientation by whole-mount EM (Gantt, 1980).

Antibody

An affinity-purified IgG fraction of a polyclonal antibody generated against a 20-kD protein isolated from the striated flagellar roots of the green flagellate *Tetraselmis striata* (for details see Salisbury et al., 1984) was used for immunolocalization and immunoblot analysis.

Electrophoretic and Immunoblot Analyses

SDS-PAGE of flagellar apparatuses of *Spermatozopsis similis* isolated in MT buffer with 2.5 mM phenylmethylsulfonyl fluoride, 30 µM pepstatin A, 40 µM leupeptin, 20 µg/ml aprotinin (the last three from Sigma Chemical Co.), and 1 mM DTT were prepared following the method of Laemmli (1970) in 15% slab gels and 0.1% SDS. Gel strips stained with Coomassie Brilliant Blue (Schleicher and Watterson, 1983) were scanned at 623 nm in a Eppendorf spectrophotometer (Brinkmann Instruments, Inc., Westbury, NY). Two-dimensional electrophoresis (first dimension: isoelectric focusing; second dimension: SDS-PAGE) was performed according to O'Farrell (1975), with a modification of ampholyte composition as described in Salisbury et al. (1984). For two-dimensional electrophoretic separations a Minigel-system (Renner, D-6701 Dannstadt, FRG) was used. For immuno-

1. *Abbreviations used in this paper*: flagellar apparatuses, isolated flagellar apparatus complexes; MT buffer, a buffer consisting of 30 mM Hepes, 5 mM $MgSO_4$, 5 mM EGTA, 25 mM KCl, pH 7.

blot analysis, proteins were electrophoretically transferred to nitrocellulose (Schleicher & Schüll BA 85, D3354 Dassel, FRG) essentially using the method of Towbin et al. (1979). Immunostaining was done according to Surek and Latzko (1984), using protein A-coated 15 nm gold particles.

Indirect Immunofluorescence

Cells were washed in fresh culture medium, placed on precleaned polylysine-coated coverslips, and allowed to adhere for 10 min. The coverslips were plunged in cold methanol (−20°C) for 3 min then rinsed (three times) in PBS. When flagellar apparatuses were used for immunofluorescence, they were prefixed in fresh 3% paraformaldehyde in MT buffer for 30 min before being allowed to adhere to polylysine-coated coverslips; methanol fixation was omitted. All samples were then quenched with 2% BSA (RIA grade; Sigma Chemical Co.) in PBS for 10 min. Specimens were then further incubated (37°C) in diluted anti-20-kD IgG (1:200) in PBS for 90 min. Coverslips were washed extensively in PBS, incubated in PBS/2% BSA for 10 min, then incubated (37°C) in either 10 μg/ml protein A/FITC or protein A/TRITC (Sigma Chemical Co.) for 60 min, washed extensively in PBS, and mounted in 0.1% *p*-phenylenediamine (Sigma Chemical Co.) in glycerine/PBS (2:1). Controls were prepared in parallel with preimmune rabbit IgG. The specimens were observed with a Zeiss IM 35 inverted microscope equipped with epi-fluorescence using either 63× or 100× oil immersion lenses. Photographs were made with Ilford HP 5 film.

Standard Electron Microscopy

Cells were prepared for thin-section EM as described by Preisig and Melkonian (1984).

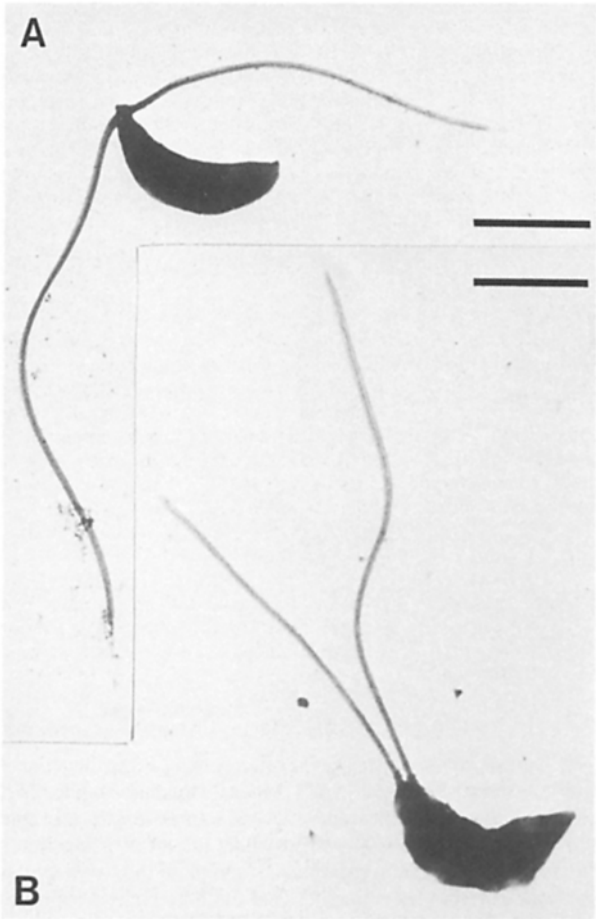

Figure 1. Whole mounts of *Spermatozopsis similis* cells stained with uranyl acetate. In *A* the flagella and basal bodies exhibit the forward swimming/antiparallel orientation while in *B* they exhibit the backward swimming/parallel orientation. Bars, 3 μm.

Immunogold Electron Microscopy

Isolated flagellar apparatuses in MT buffer or 10^{-6} M Ca^{2+} (see above) were fixed by adding an equal volume of 6% formaldehyde and 0.5% glutaraldehyde in MT buffer on ice for 60 min. The flagellar apparatuses were sedimented (Biofuge B; 1,000 *g* for 5 min) and resuspended in PBS (three times) using a 1-ml tissue homogenizer tube. Flagellar apparatuses were then incubated (37°C) with agitation in PBS/1% BSA for 60 min. An equal volume of anti-20-kD IgG (1:200 dilution in PBS) was added directly to the samples (control samples were prepared using preimmune rabbit IgG) and further incubated for 60 min. The flagellar apparatuses were washed in PBS/1% BSA (three times) by repeated centrifugation and resuspension (as above), then transferred to colloidal gold (15 nm) conjugated to protein A (Surek and Latzko 1984), and incubated as above for 60 min. The flagellar apparatuses were washed in PBS/1% BSA (three times), and the final pellet postosmicated in 1% OsO_4 in MT buffer. Pellets were washed twice in MT buffer, transferred to agar, dehydrated, embedded, sectioned, and stained as described by McFadden and Melkonian (1986).

Results

Flagellar Apparatus Ultrastructure of S. similis

The naked cells of the green flagellate *Spermatozopsis similis* are crescent shaped (~6 μm long) and spirally twisted (Fig. 1). Two sub-equal flagella project laterally (basal bodies antiparallel) from the cell anterior during forward swimming and rest (Fig. 1 *A*). The flagellar basal apparatus resembles that of *Chlamydomonas reinhardtii* comprising four microtubular roots (Fig. 3 *E*), two of which are accompanied by finely striated fibers (System I fibers; defined in Melkonian, 1980*b*). Rhizoplasts (System II fibers; defined in Melkonian, 1980*b*) have not yet been detected in thin section material. The two basal bodies are interconnected by one distal and two proximal connecting fibers. The distal connecting fiber shows no cross-striations, being composed of a homogeneous bundle of filaments of 3–8-nm diameter (Fig. 9). (For a detailed ultrastructural analysis of *S. similis* see Melkonian and Preisig, 1984, and Preisig and Melkonian, 1984.)

Responses of S. similis to Light Shock

Cells of *S. similis* swim forward in the breaststroke manner with the flagella describing an asymmetrical beat form (cf.

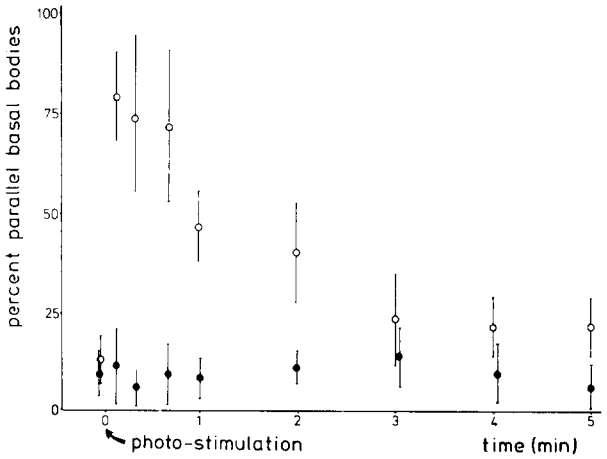

Figure 2. Effect of photostimulation on basal body orientation in *Spermatozopsis similis* cells in 10^{-9} M Ca^{2+} (●) and 10^{-4} M Ca^{2+} (○). The circles show the mean percentage ($n > 100$) of cells having parallel orientated basal bodies. The bars show the standard deviation (five different experiments).

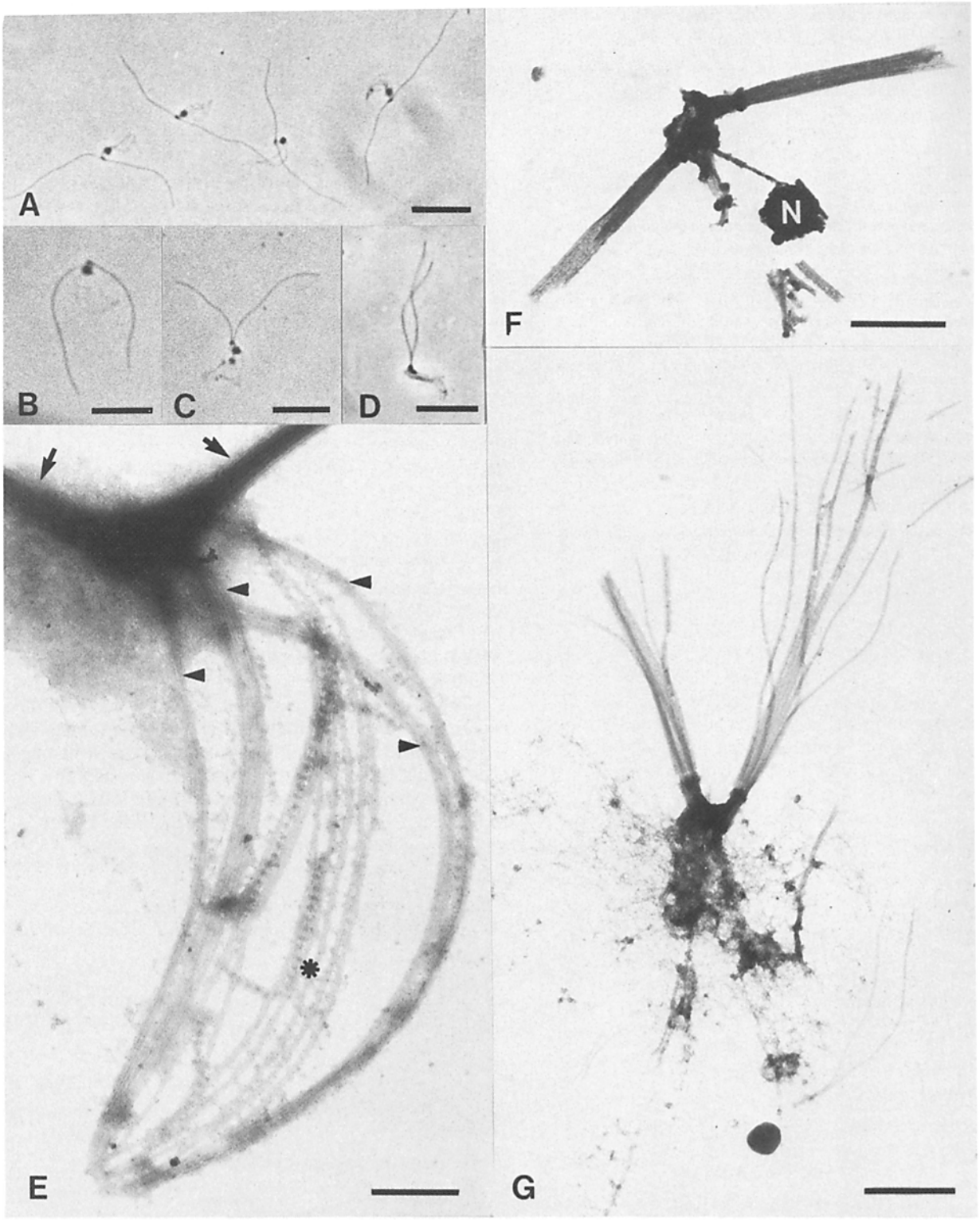

Figure 3. Isolated detergent-lysed flagellar apparatuses of *Spermatozopsis similis*. (*A*) Phase contrast light micrograph of four flagellar apparatuses in MT buffer illustrating the antiparallel basal body orientation. (*B*) When resuspended in 10^{-9} M Ca^{2+}, the basal bodies of the flagellar apparatus remain in the antiparallel configuration. (*C*) In 10^{-6} M Ca^{2+} the basal bodies and attached axonemes are reoriented to the parallel configuration. (*D*) Flagellar apparatus resuspended in 10^{-5} M Ca^{2+}. (*E*) Negatively stained whole mount of a flagellar apparatus isolated in MT buffer without $MgSO_4$ or KCl resulting in loss of the nucleus. Remaining components comprise the two axonemes (*arrows*) attached to the interconnected basal bodies, the secondary cytoskeletal microtubules (*asterisk*), and the four microtubular roots (*arrowheads*). The latter remain in their in vivo positions, following the cell contours and converging at the cell posterior. (*F*) Negatively stained whole mount of a flagellar apparatus with the axonemes mechanically amputated in MT buffer illustrating remaining stumps of the axonemes (basal body orientation antiparallel) and microtubular roots plus a remnant of the nucleus (*N*). (*G*) Flagellar apparatus prepared as in *F*, then resuspended in 10^{-6} M Ca^{2+}. The basal bodies and axoneme remnants assume the parallel orientation. Bars: (*A*) 10 μm; (*B–D*) 5 μm; (*E*) 500 nm; (*F* and *G*) 1 μm.

Ringo, 1967; Hyams and Borisy, 1978; Brokaw et al., 1982). When cells are photostimulated in growth medium, ~70% perform the photophobic response, either darting backward for a distance of ~20 μm or stopping momentarily before resuming forward swimming after 1–2 s. During backward swimming the flagella project directly anteriorly (i.e., parallel to one another) from the cell (Fig. 1 *B*), and beat in the symmetrical, undulating form (cf. Bessen et al., 1980; Hyams and Borisy, 1978).

Cells resuspended in Ca^{2+}-buffered solutions swim forward normally and at Ca^{2+} concentrations $\geqslant 10^{-5}$ M photostimulation induces the photophobic response. However, no cell in solutions containing $<10^{-5}$ M Ca^{2+} reacts to the photostimulation with an observable photophobic response.

Fig. 2 shows the effect of photostimulation on basal body orientation at two different Ca^{2+} concentrations (10^{-9} and 10^{-4} M). Before photostimulation ~15% of the cells in 10^{-4} M Ca^{2+} have parallel-oriented basal bodies (Fig. 2). Directly after photostimulation this percentage increases to 80%. Although the cells resume forward swimming a few seconds after stimulation, examination of the fixed material indicates that the restoration of the antiparallel basal body configuration only occurs after ~3–4 min. No basal body reorientation is induced in photostimulated cells in 10^{-9} M Ca^{2+} (Fig. 2).

Isolation and Structure of Flagellar Apparatuses

The detergent-lysed isolated flagellar apparatuses of *Spermatozopsis similis* comprise the two axonemes, basal bodies, connecting fibers, microtubular and fibrous roots, secondary cytoskeletal microtubules, and the demembranated nucleus (Figs. 3, 8, and 9). The microtubular flagellar roots in flagellar apparatuses of *S. similis* outline the original contours of the cell and converge at the posterior end (Fig. 3 *E*). In *S. similis* the axonemes invariably project laterally (antiparallel) in MT buffer (Figs. 3, *A* and *E*, and 9, *B*, *C*, and *G*). The flagellar apparatuses remain structurally intact in cold MT buffer for at least 24 h. If $MgSO_4$ and KCl are omitted from the MT buffer, the result is the loss of the nucleus (Fig. 3 *E*) and splaying of the axonemal doublets (not shown). In contrast to previous findings using *Chlamydomonas* (Hyams and Borisy, 1978; Kamiya and Witman, 1984), we experienced no difficulties in retaining the flagella during preparation of flagellar apparatuses from *S. similis*.

Reactivation of Flagellar Apparatuses from Spermatozopsis similis

A very high percentage (routinely >90%) of flagellar apparatuses could be reactivated in vitro up to at least 6 h after isolation, and flagellar beating in reactivated apparatuses sometimes persisted up to 90 min. Both flagellar beat form and basal body orientation depend on the Ca^{2+} concentration as illustrated in Fig. 4. At $\leqslant 10^{-8}$ M Ca^{2+} all basal bodies are oriented antiparallel (Fig. 4) and all axonemes beat in the asymmetrical form. At 10^{-7} M Ca^{2+} 90% of the basal bodies undergo reorientation to the parallel position (the remaining basal bodies are oriented antiparallel or in intermediate positions) and 30% of the axonemes beat in the symmetrical beat form (Fig. 4). At or above 10^{-6} M Ca^{2+} all basal bodies are reoriented anteriorly (i.e., to the parallel configuration) and all axonemes adopt the symmetrical beat form (Fig. 4). At 10^{-4} M Ca^{2+} the axonemes detach from the flagellar apparatuses and swim individually in the symmetrical mode.

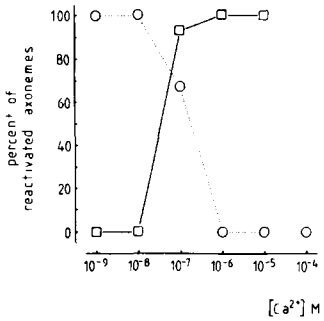

Figure 4. Effect of different Ca^{2+} concentrations on isolated reactivated flagellar apparatuses of *Spermatozopsis similis*. The percentage of reactivated axonemes beating with an asymmetrical (○) waveform, and the percentage of flagellar apparatuses with parallel-oriented basal bodies (□) is compared.

Basal Body Reorientation Is Independent of Flagellar Beat

By viewing flagellar apparatuses adhering to a slide but with both flagella free to beat, and changing the reactivation solutions in the perfusion chamber, it is possible to assess the effect of changes in Ca^{2+} levels on both the flagellar beat form and the basal body orientation simultaneously. When 10^{-9} M Ca^{2+} is replaced by 10^{-6} M Ca^{2+}, the axonemes convert to the symmetrical beat form and simultaneously the basal bodies rapidly reorient to the parallel configuration. When 10^{-9} M Ca^{2+} is reintroduced, the axonemes readopt the asymmetrical beat form. However, even though the change in axonemal beat form is reversible, the basal bodies remain in the parallel configuration and do not return to the antiparallel configuration (Fig. 5 *A*). These observations were further verified by centrifuging flagellar apparatuses reactivated in 10^{-6} M Ca^{2+} and resuspending them in reactivation solutions containing 10^{-9} M Ca^{2+}. Again, the flagellar beat form is reversed, but basal bodies do not resume the antiparallel configuration. These flagellar apparatuses were monitored for 60 min during which time the axonemes continue to beat in the asymmetrical form but the basal bodies retain a parallel orientation.

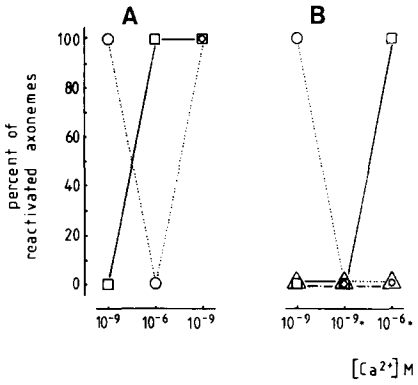

Figure 5. Effect of altering the Ca^{2+} concentration and/or introducing orthovanadate on the axonemal beat form and basal body orientation in flagellar apparatuses of *Spermatozopsis similis*. The percent of axonemes beating with asymmetrical (○) or symmetrical (△) waveforms, plus the percent having parallel-oriented basal bodies (□) in the same flagellar apparatus is shown. In *A*, the reactivation solution in the perfusion chamber is changed from 10^{-9} M Ca^{2+} to 10^{-6} M Ca^{2+}, then back to 10^{-9} M Ca^{2+}. In *B*, 10^{-9} M Ca^{2+} is replaced by 10^{-9} Ca^{2+} plus 1 μM orthovanadate (*), then by 10^{-6} M Ca^{2+} plus 1 μM orthovanadate (*).

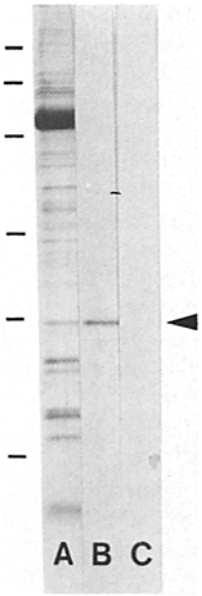

Figure 6. SDS-PAGE analysis of isolated flagellar apparatuses of *Spermatozopsis similis*. (*A*) Amido black–stained nitrocellulose blot of flagellar apparatuses isolated in MT buffer. (*B*) Nitrocellulose blot of flagellar apparatuses labeled with 20-kD antibody and protein A–colloidal gold showing an antigenic band of M_r 20,000. (*C*) Nitrocellulose blots of flagellar apparatuses incubated in preimmune IgG and protein A–colloidal gold illustrate no labeling. Molecular weight markers indicated at left are from top to bottom: phosphorylase b (97,400); BSA (67,000); ovalbumin (45,000); carbonic anhydrase (29,000); trypsin inhibitor (20,100); α-lactalbumin (14,400).

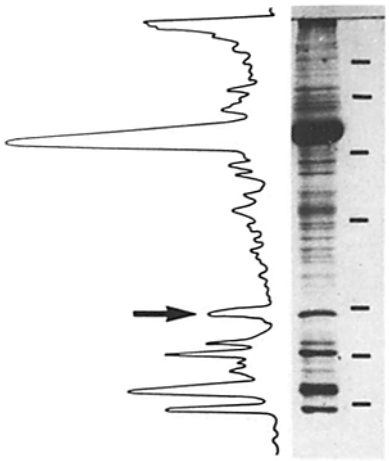

Figure 7. Densitometric scan of an SDS gel (15%) of isolated flagellar apparatuses from *Spermatozopsis similis* stained with Coomassie Brilliant Blue. The antigenic 20-kD protein (*arrow*) comprises ∼3% of the detected proteins. In this experiment no protease inhibitors were included during isolation of flagellar apparatuses. Molecular weight markers as indicated at left are from top to bottom: phosphorylase b (97,400); BSA (67,000); ovalbumin (45,000); carbonic anhydrase (29,000); trypsin inhibitor (20,100); myoglobin (17,200); cytochrome c (12,300).

When orthovanadate (1 μM) is added to 10^{-9} M Ca^{2+} and exchanged for 10^{-9} M Ca^{2+} in the perfusion chamber, the axonemes cease beating (Fig. 5 *B*). If the 10^{-9} M Ca^{2+} including orthovanadate is then replaced by 10^{-6} M Ca^{2+} also including 1 μM orthovanadate, the axonemes remain paralyzed but the basal bodies instantly reorient to the parallel orientation (Fig. 5 *B*). The same responses were observed in flagellar apparatuses that were initially reactivated in 10^{-9} M Ca^{2+} and the reactivation solutions changed by centrifugation and resuspension.

If DTT is omitted from the original isolation buffer, axonemes could not be reactivated with ATP. However, when the Ca^{2+} concentration is increased to 10^{-6} M, basal bodies reorient to the parallel configuration. This reorientation could also be induced by 10^{-6} M Ca^{2+} in reactivation solutions lacking ATP (Figs. 3 *C* and 9, *D* and *E*). Ca^{2+}-induced reorientation of basal bodies is also observed in preparations of flagellar apparatuses isolated in MT buffer without $MgSO_4$ and KCl. These flagellar apparatuses have splayed axonemal doublets and the nucleus is lost, i.e., no nucleus–basal body connector is present anymore.

When basal body/connecting fiber units (sometimes the proximal portions of axonemes remain attached to the basal bodies; Fig. 3, *F* and *G*) produced by homogenization of flagellar apparatuses in MT buffer (Fig. 3 *F*) are resuspended in 10^{-6} M Ca^{2+} without ATP, the basal bodies reorient to the parallel configuration (Fig. 3 *G*).

Electrophoretic Analyses

Immunoblots of isolated flagellar apparatuses of *S. similis* as separated by SDS-PAGE (15%) and labeled with anti–20-kD IgG detect a single antigenic polypeptide of M_r 20,000 (Fig. 6). Photometric scans of Coomassie Blue–stained gel strips indicate that the antigenic 20-kD polypeptide is a significant component of the flagellar apparatuses representing ∼3% of the Coomassie Brilliant Blue–stained material (see *arrow* in Fig. 7). Two-dimensional PAGE of flagellar apparatuses of *S. similis* separates the antigenic 20-kD polypeptide into two acidic isoforms, both immunoreactive with the anti–20-kD IgG (not shown).

Immunolocalization

In whole cells of *S. similis* immunofluorescent labeling is observed around the proximal region of the flagella (Fig. 8 *A*). In most instances the immunofluorescent labeling could be resolved into a larger anterior, horizontally oriented rod and a smaller posteriorly directed band, triangle, or dot (Fig. 8, *B* and *D*). In isolated flagellar apparatuses of *S. similis*, a similar pattern of anti–20-kD immunofluorescence is present but the more anterior zone is bipartite and the posterior region more compact (Fig. 8 *H*). Preimmune controls of *S. similis* exhibited no fluorescence (not shown).

Immunogold EM localization of antigenicity in the flagellar apparatuses of *S. similis* shows extensive and homogeneous labeling over the entire exposed surface of the distal connecting fiber (Fig. 9, *B–E*). In flagellar apparatuses treated with 10^{-6} M Ca^{2+} the basal bodies are reoriented to the parallel configuration and the connecting fiber is more condensed (Fig. 9, *D* and *E*). The distal connecting fiber of reoriented basal bodies also exhibits gold labeling at its surface (Fig. 9, *D* and *E*). A filamentous connector linking the basal bodies to the nucleus is also immunoreactive (Fig. 9 *F*). Other structures of the flagellar apparatuses (i.e., axonemes, microtubular roots, proximal connecting fibers, basal bodies, System I fibers, and nuclear matrix) are not labeled with gold particles (Fig. 9, *B–F*). In preimmune controls the distal connecting fiber and other parts of the flagellar apparatuses are not labeled (Fig. 9 *G*).

Discussion

Basal Body Reorientation: An Independent Motility System

In *Spermatozopsis similis*, and certain other flagellated green algae, a reorientation of basal bodies accompanies the switch from forward to reverse swimming during the photophobic response (Watson, 1975; Melkonian, 1978; Melkonian and

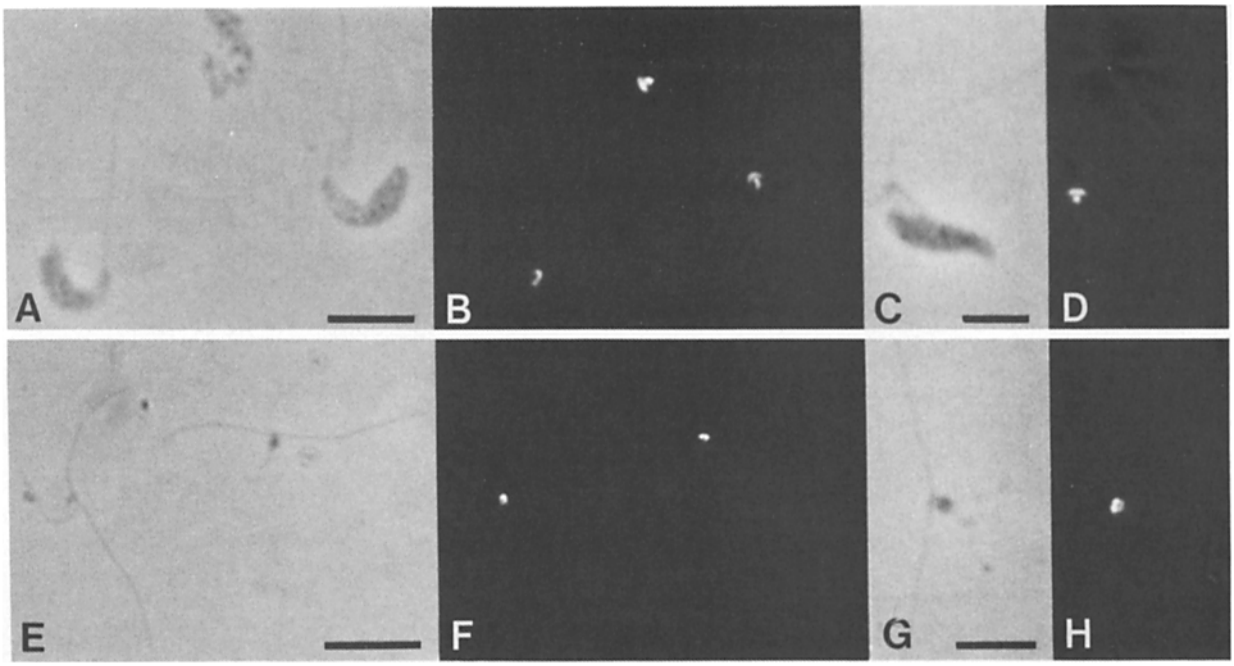

Figure 8. Indirect immunofluorescence of *Spermatozopsis similis*. (*A*) Phase contrast micrograph of three cells. (*B*) Immunofluorescence of the cells depicted in *A* showing two antigenic sites in the cell anterior near the basal body region. (*C*) Phase contrast micrograph of a cell at higher magnification. (*D*) The anti-20-kD fluorescence of the cell depicted in *C* can be resolved into two regions: a broader rod-shaped anterior zone and a more posteriorly situated dot. (*E*) Phase contrast of two flagellar apparatuses with basal bodies in antiparallel orientation. (*F*) Immunofluorescence in isolated flagellar apparatuses depicted in *E* illustrating anti-20-kD fluorescence at the basal body region. (*G*) Phase contrast micrograph of a single flagellar apparatus. (*H*) The anti-20-kD immunofluorescence of the flagellar apparatus shown in *G* is bipartite. Bars: (*A*) 5 μm; (*C*) 3 μm; (*E*) 10 μm; (*G*) 4 μm.

Preisig, 1984). The in vivo reaction of *S. similis* to step up photostimulation in Ca^{2+}-buffered media indicates that reverse swimming and basal body reorientation are dependent on external Ca^{2+}. Ca^{2+} has previously been shown to be essential for the change of flagellar beat form during the photophobic response in *Chlamydomonas* (Schmidt and Eckert, 1976). It is widely held that step up photostimulation in *Chlamydomonas* initiates transmembrane ion fluxes leading to an increase in intracellular Ca^{2+} (Schmidt and Eckert, 1976; Hyams and Borisy, 1978; Bessen et al., 1980). The behavior of reactivated, isolated flagellar apparatuses of *S. similis* in response to different Ca^{2+} concentrations is in good agreement with previous observations of detergent-extracted, reactivated flagellar apparatuses of *Chlamydomonas reinhardtii* (Hyams and Borisy, 1978) with $\geqslant 10^{-7}$ M Ca^{2+} altering the form of flagellar beat to the symmetrical mode. Although we used the reactivation solutions of Bessen et al. (1980), the free Ca^{2+} necessary to cause axonemes to switch to symmetric beating was between one and two orders of magnitude lower than observed in isolated *Chlamydomonas* axonemes by Bessen et al. (1980). These authors reported that isolated *Chlamydomonas* axonemes were quiescent at 10^{-6} and 10^{-5} M Ca^{2+}, a situation that was not observed in reactivated flagellar apparatuses of *S. similis*. This suggests that axonemes of *Chlamydomonas* perhaps loose some regulatory components during the isolation procedure and that there is a real difference between isolated axonemes and isolated flagellar apparatuses. In vitro reactivation of the flagellar apparatuses of *S. similis* shows that about the same Ca^{2+} levels (or perhaps even slightly lower Ca^{2+} levels; Fig. 4) also trigger rapid basal body reorientation. Although basal body reorientation and conversion of axonemal beat form occur at about the same level of free Ca^{2+}, our data demonstrate that reorientation is independent of flagellar beating. Firstly, Ca^{2+}-induced basal body reorientation occurs in flagellar apparatuses of *S. similis* in which flagellar beating is blocked. Secondly, Ca^{2+}-induced reorientation of basal bodies takes place in basal body/connecting fiber complexes in which the axonemes have been amputated. These data indicate that the motor for Ca^{2+}-induced basal body reorientation during the photophobic response is independent of flagellar motility and does not reside in the axoneme. Thus, two separate motility systems respond with similar thresholds to the same stimulus, thereby leading to a coordinated change in the direction of swimming in *S. similis*.

The Distal Connecting Fiber: A Ca^{2+}-modulated Contractile Structure

In this study we have shown by SDS-PAGE and immunoblotting that isolated flagellar apparatuses of *S. similis* contain a 20-kD polypeptide that exhibits immunoreactivity to an antibody generated against the 20-kD Ca^{2+}-modulated contractile protein from striated flagellar roots of *Tetraselmis striata* (Salisbury et al., 1984). In *Tetraselmis striata* and *Chlamydomonas reinhardtii* the 20-kD protein exists in two isoforms with slightly different isoelectric points (Salisbury et al., 1984; Wright et al., 1985); the more acidic of which is phosphorylated (Salisbury et al., 1984). In flagellar apparatuses of *S. similis* the 20-kD antigenic polypeptide also consists of two immunoreactive isoforms (unpublished results). The immunoreactivity in flagellar apparatuses of *S.*

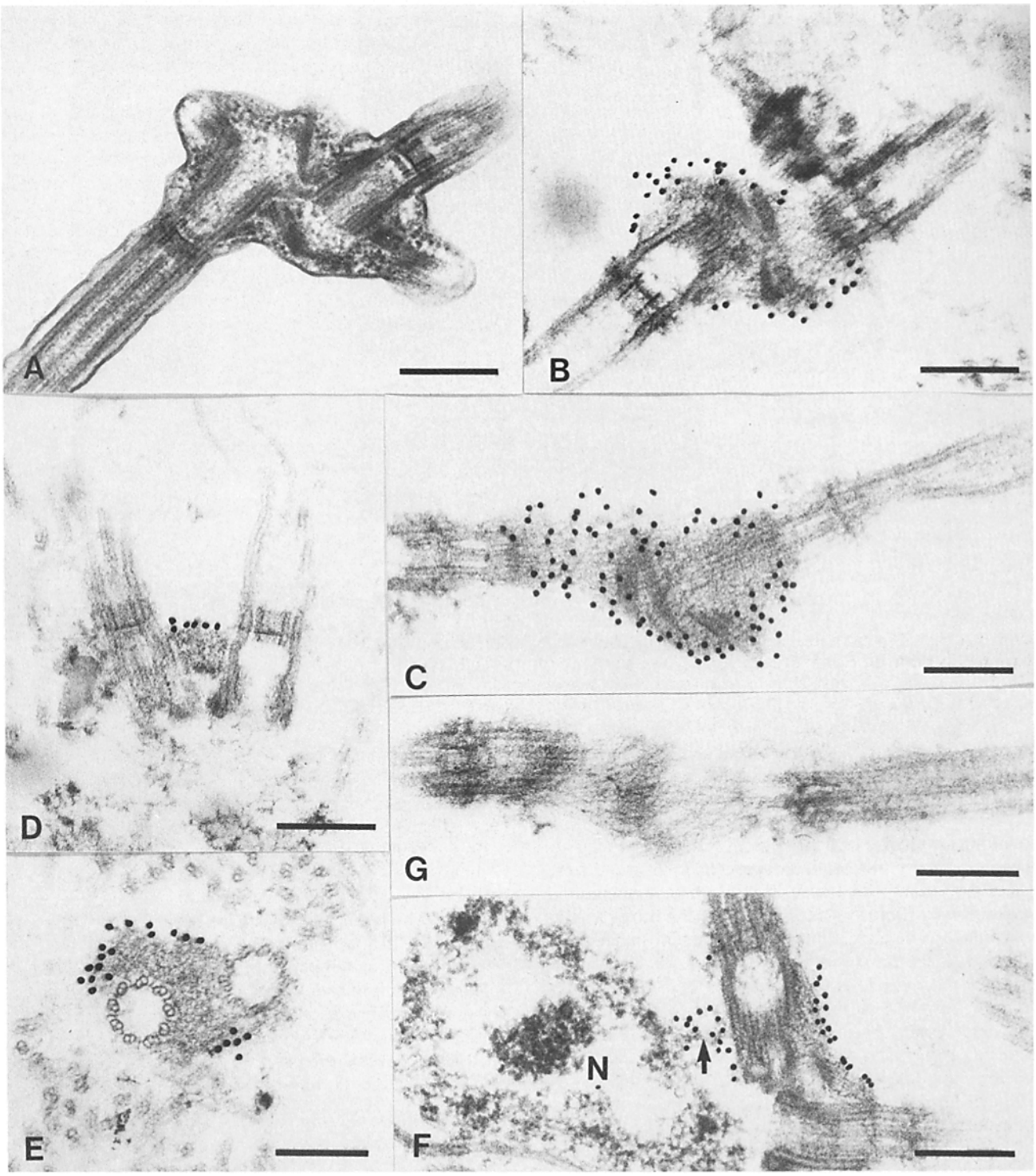

Figure 9. Ultrastructural localization of 20-kD protein in *Spermatozopsis similis* by immunogold labeling of isolated flagellar apparatuses. (*A*) Cross section of anterior-most portion of a whole cell showing the basal bodies in the antiparallel orientation. (*B*) Immunogold-labeled isolated flagellar apparatus (MT buffer) demonstrating the same basal body orientation. The exposed surface of the distal connecting fiber is immunoreactive. (*C*) A section at a more anterior level than *B*, shows homogeneous labeling over the entire surface of the distal connecting fiber. (*D*) In flagellar apparatuses resuspended in 10^{-6} M Ca^{2+}, the basal bodies are oriented parallel and the distal connecting fiber is condensed. Again, the exposed surface of the distal connecting fiber is immunoreactive. (*E*) A cross section through a pair of basal bodies in the parallel orientation with surface labeling of the distal connecting fiber. The distal connecting fiber extends around at least six of the doublets/triplets on each axoneme/basal body. (*F*) A fibrous connector (*arrow*) between the nucleus (*N*) and basal bodies is also immunoreactive. (*G*) In the preimmune control a glancing section of the distal connecting fiber comparable to *C* has no gold labeling. Bars, 300 nm.

similis has been localized by indirect immunofluorescence and immunogold EM to two structures: the distal connecting fiber, and the nucleus–basal body connector. The entire exposed surface of the distal connecting fiber in *S. similis* showed extensive immunogold labeling suggesting that the 20-kD polypeptide is a major component of the distal connecting fiber in this organism.

We conclude that the distal connecting fiber of *S. similis* is a Ca^{2+}-modulated contractile organelle that is involved in basal body reorientation during the photophobic response in this organism (Fig. 10). The nucleus–basal body connector is probably not involved in basal body reorientation since basal body reorientation occurs in flagellar apparatuses when the nucleus and most of the flagellar roots are removed (i.e., in basal body/connecting fiber complexes). In addition we note that the extended distal connecting fiber subtends the plasmamembrane (Melkonian and Preisig, 1984), and is therefore in close proximity to the putative Ca^{2+} influx site.

Reextension of the Distal Body Connecting Fiber

The mechanism by which the basal bodies resume the antiparallel configuration has not been determined. From the observations presented here, it is clear that reduction of free Ca^{2+}, albeit sufficient to restore the asymmetrical beat form of the axonemes, is insufficient to reestablish the antiparallel basal body configuration. Conversely we can deduce that axonemal movement during forward swimming does not in itself cause basal bodies to regain the antiparallel configuration. The observation that living cells resume forward swimming while the basal bodies are still oriented parallel after a photophobic response corroborates this conclusion. At present we assume that some factor(s) critical to the mechanism of distal connecting fiber reextension is either lacking or inoperant in our in vitro system.

Our observations of a rapid contraction followed by a relatively prolonged reextension period (in the range of 1–3 min) are the first kinetic data relating to the contraction/extension cycle for an algal contractile organelle containing the 20-kD protein. In the ciliated vorticellids a contractile stalk, the spasmoneme, also undergoes a rapid contraction and then slowly reextends (Amos, 1971). A 20-kD protein (spasmin; Amos et al., 1975) has been identified as a major component of the spasmoneme and shares many properties with the algal 20-kD protein (Salisbury et al., 1984). Amos (1971) suggested that spasmoneme extension was the energy-requiring part of the contraction/extension cycle, and Salisbury et al. (1984) have concluded that the 20-kD protein of *T. striata* flagellar roots undergoes phosphorylation during the extension cycle. It is therefore possible that the relatively slow extension of these contractile organelles is directly related to a protein phosphorylation step. Interestingly, ATPase activity has been localized at the ultrastructural level to cross-bands of striated flagellar roots in *Tetraselmis* (Salisbury, 1983) and to certain cross-bands of the distal connecting fiber of the colorless chlamydomonad flagellate *Polytomella* (White and Brown, 1981).

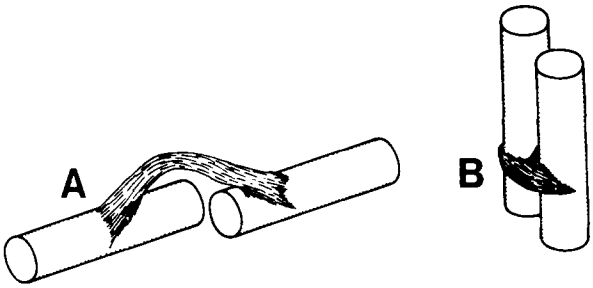

Figure 10. A schematic representation of the basal bodies and distal connecting fiber in *Spermatozopsis similis*. During forward swimming the basal bodies are oriented antiparallel at an angle of 180° to one another (*A*), but during backward swimming the basal bodies are in a parallel orientation (*B*). It is proposed that the rapid reorientation into the position shown in *B* is induced by a Ca^{2+}-modulated contraction of the distal connecting fiber during the photophobic response.

Physiological Significance of Basal Body Reorientation in Green Flagellates

Basal body reorientation accompanies a photophobic response (a switch between asymmetric and symmetric flagellar beating) in *Spermatozopsis similis* and numerous green algal zoospores and gametes. In these organisms basal bodies assume an angle of 180° to each other (antiparallel orientation) during forward swimming. Whereas the significance of the antiparallel orientation of basal bodies in these algae is not yet clear (a more powerful breast stroke?), it is likely that basal body reorientation to the parallel orientation facilitates the switch from forward to backward swimming. In green flagellates with a more or less fixed basal body angle (*Chlamydomonas reinhardtii*, *Dunaliella bioculata*, *Chlorogonium elongatum*; Ringo, 1967; Hyams and Borisy, 1978; Hoops and Witman, 1985; Marano et al., 1985) a sharp reverse bend develops near the flagellar bases as the flagella become parallel during the transition from forward to backward swimming. In contrast to the distal connecting fiber of *S. similis*, the distal connecting fiber in these flagellates is distinctly cross-striated and labels much less extensively with the anti–20-kD antibody (unpublished results). This suggests that the Ca^{2+}-modulated contractile protein is probably only a minor component of the distal connecting fiber in these flagellates and that this may be the reason for the lack of a distinctive distal connecting fiber contraction/extension cycle in, e.g., *Chlamydomonas* and *Dunaliella*. Additionally, an apparently normal photophobic response occurs in a *C. reinhardtii* mutant lacking the distal connecting fiber (Hoops et al., 1984).

To our knowledge basal body reorientation mediated by the contraction of a fiber connecting the basal bodies is the first example of a nondynein, photo-induced motility response in flagellated algae, and establishes a first physiological function for the 20-kD striated flagellar root protein.

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