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# ATP Production in *Chlamydomonas reinhardtii* Flagella by Glycolytic Enzymes

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**Eukaryotic cilia and flagella are long, thin organelles, and diffusion from the cytoplasm may not be able to support the high ATP concentrations needed for dynein motor activity. We discovered enzyme activities in the *Chlamydomonas reinhardtii* flagellum that catalyze three steps of the lower half of glycolysis (phosphoglycerate mutase, enolase, and pyruvate kinase). These enzymes can generate one ATP molecule for every substrate molecule consumed. Flagellar fractionation shows that enolase is at least partially associated with the axoneme, whereas phosphoglycerate mutase and pyruvate kinase primarily reside in the detergent-soluble (membrane + matrix) compartments. We further show that axonemal enolase is a subunit of the CPC1 central pair complex and that reduced flagellar enolase levels in the *cpc1* mutant correlate with the reduced flagellar ATP concentrations and reduced in vivo beat frequencies reported previously in the *cpc1* strain. We conclude that in situ ATP synthesis throughout the flagellar compartment is essential for normal flagellar motility.**

## INTRODUCTION

The motor enzymes responsible for the beating of eukaryotic cilia/flagella, the dynein ATPases, are distributed uniformly along the nine outer doublet microtubules that form the core of this organelle. ATP hydrolysis is also required for activity of the cytoplasmic dynein and kinesin motors that drive intraflagellar transport (IFT; Rosenbaum and Witman, 2002). IFT brings axonemal precursors from the cell body to the flagellar tip for assembly and turnover products from the tip back to the cell body for recycling (Qin *et al.*, 2004). To power both flagellar beating and IFT, ATP must be readily available throughout a long, thin flagellar compartment that has a restricted opening to the cytoplasm (Figure 1).

ATP diffusing into flagella from the cytoplasm should form a steep concentration gradient, and therefore distal regions of flagella would be starved for ATP unless diffusion rates significantly exceed hydrolysis rates. The sperm flagella of mammals have surmounted this problem by localizing mitochondria and glycolytic enzymes to the flagellar compartment and by supplying glycolytic enzymes with fermentable sugars directly from seminal fluid (Lardy and Phillips, 1941; Mukai and Okuno, 2004). ATP is probably generated in situ along the entire length of sperm flagella (Mohri *et al.*, 1965), but relatively little is known about mechanisms that anchor or localize glycolytic enzymes within sperm. Targeted knockout of a sperm-specific glycolytic enzyme (glyceraldehyde 3-phosphate dehydrogenase-S) normally anchored to the fibrous sheath (Westhoff and Kamp, 1997) results in substantial decreases in ATP levels and sperm motility (Miki *et al.*, 2004). Although diffusion can adequately disperse ATP that is synthesized by mitochondria in spermatozoa (Nevo and Rikmenspoel, 1970), in other cell types there are structures in the transition zone between basal bodies and flagella (Figure 1) that likely form a diffusion barrier between cytoplasmic and flagellar compartments. Glycolytic enzyme activity has not been reported in motile cilia and flagella other than sperm tails, but has been detected in at least some types of nonmotile cilia, such as the outer segments of mammalian photoreceptor cells (Hsu and Molday, 1991). In situ ATP generation by glycolytic enzymes in these highly modified primary cilia has been proposed to provide energy for the continued synthesis of cGMP essential for phototransduction (Hsu and Molday, 1994). In some organisms, energy for ciliary activity may also be provided by phosphate shuttles, such as phosphocreatine/creatine phosphokinase in sea urchin (Tombes *et al.*, 1987) and mammalian (Huszar *et al.*, 1997) spermatozoa, and in chicken photoreceptor outer segments (Wallimann *et al.*, 1986), and phosphoarginine/arginine phosphokinase in *Paramecium* cilia (Noguchi *et al.*, 2001), but these shuttles have not been reported in motile cilia and flagella of metazoan cells other than spermatozoa.

No mechanisms for in situ ATP synthesis have been reported in flagella of the model organism *Chlamydomonas reinhardtii*, and neither creatine kinase nor arginine kinase has been detected in *C. reinhardtii* flagella (Watanabe and Flavin, 1976). Adenylate kinase activity, on the other hand, has been demonstrated not only in mammalian sperm, but also in cilia and flagella from a variety of lower eukaryotes including *C. reinhardtii*, and some of these enzymes are

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Abbreviations used: GAP, glyceraldehyde 3-phosphate dehydrogenase; IFT, intraflagellar transport.

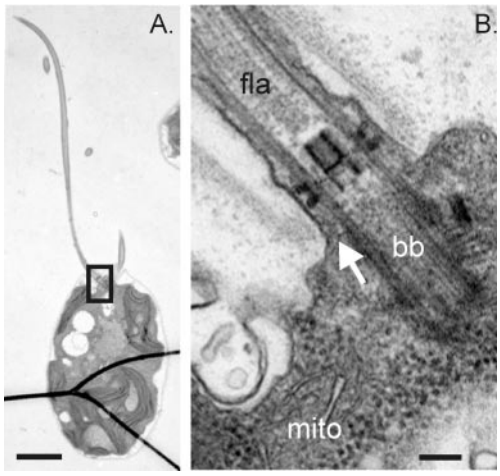


**Figure 1.** Electron micrographs illustrating the restricted pathway for diffusion of ATP from the cell body into the flagellar compartment. (A) Thin section through a *Chlamydomonas* cell body and one of the two flagella. Boxed region in A is enlarged in B, which shows that ATP synthesized by mitochondria (mito) must pass the basal body (bb) to the flagellum (fla) through a transition zone that links flagellar microtubules to the cell membrane (arrow). Bar, (A) 2 $\mu$m, (B) 100 nm.

anchored to the axoneme (Watanabe and Flavin, 1976; Schoff *et al.*, 1989; Nakamura *et al.*, 1999; Wirschell *et al.*, 2004; Zhang and Mitchell, 2004; Ginger *et al.*, 2005). Knockout mice lacking adenylate kinase activity show decreased efficiency of cellular energetics (Janssen *et al.*, 2000), suggesting that at least in some cases this enzyme is important for efficient energy metabolism. However, adenylate kinases are thought to work primarily to maintain a constant adenylate charge, the ratio between ATP and ADP+AMP, rather than a constant ATP concentration. Although flagellar adenylate kinases presumably play an important role in the overall functioning of the organelle, at least some of the flagellar isoforms appear to have no direct effect on flagellar beating. The *oda5* and *oda10 C. reinhardtii* mutants show reduced flagellar adenylate kinase activity in addition to lacking outer dynein arms (Wirschell *et al.*, 2004); however, these cells have the same motility characteristics as all the other *oda* strains (Kamiya, 1988), which also lack outer dynein arms, but have normal adenylate kinase activity. Likewise, RNAi knockdown of trypanosome flagellar adenylate kinase activity has no apparent effect on motility in that organism (Pullen *et al.*, 2004). Thus the presence of adenylate kinases may be important for normal nucleotide metabolism in cilia and flagella, but additional sources of ATP may also be necessary to maintain the high ATP levels needed for the continuous motility typical of these organelles.

We report here that *C. reinhardtii* flagella possess three enzymes of the lower half of the glycolytic pathway, which allow ATP production in situ from the glycolytic intermediate 3-phosphoglycerate. In addition, we show that one of these enzymes, enolase, is linked to the 9 + 2 microtubule scaffold (axoneme) through its association with the CPC1 central pair protein. Reductions in flagellar enolase in *cpc1* mutants correlate with reductions in motility due to reduced intraflagellar ATP concentrations. Finally, we show that the other two glycolytic enzymes, phosphoglycerate mutase and an unusual pyruvate kinase, are located in the membrane + matrix fraction.

## MATERIALS AND METHODS

*C. reinhardtii* wild-type strain 137c (CC 124) and central pair assembly defective strains *pf15*, *pf18*, *pf19*, and *pf20* were obtained from Elizabeth Harris at the *C. reinhardtii* Genetics Center, Duke University. Mutant strain *cpc1-2* (CC 3708) was described previously (Mitchell and Sale, 1999). Methods for electron microscopy of whole cells have been described previously (Mitchell, 2003).

### *Flagellar Isolation and Fractionation*

Unless otherwise indicated, cells grown in acetate-enriched medium (Sager and Granick, 1953) were suspended in HMDS (10 mM HEPES, 5 mM $MgSO_4$, 1 mM dithiothreitol [DTT], 5% sucrose, pH 7.4) and deflagellated with 5 mM dibucaine (Witman, 1986). Flagella collected by differential centrifugation were demembranated by being resuspended in HMDEK (30 mM HEPES, 5 mM $MgSO_4$, 1 mM DTT, 0.5 mM EGTA, 25 mM potassium acetate, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and mixed with an equal volume of HMDEK containing 0.5% NP-40 (Fluka 74385, Sigma-Aldrich, St. Louis, MO). The detergent-soluble fraction was separated from the detergent insoluble axonemes by centrifugation at 14,000 $\times$ *g* for 10 min at 4°C. For some experiments, the flagellar matrix was released by freeze-thaw treatment. Flagella suspended in HMDEK were frozen in a dry ice-ethanol bath, stored at −70°C for up to 24 h, thawed on ice, and spun as above to separate the soluble matrix fraction from the pelletable axoneme + membrane fraction. The insoluble fraction was resuspended to the same volume in HMDEK (freeze-thaw preparations) or HMDEK + NP-40 (NP-40 preparations) before enzyme assay or gel sample preparation. For experiments used to purify fractions for enzyme assays, the HMDS and HMDEK solutions contained 0.1 mM $NAD^+$ to stabilize glyceraldehyde 3-phosphate dehydrogenase activity. To prepare a cytoplasmic extract, deflagellated cell bodies were resuspended in an equal volume of HMDEK, frozen in dry ice-ethanol, thawed, and centrifuged at 14,000 $\times$ *g* for 10 min at 4°C. The soluble fraction was used for enzyme assays.

To solubilize the CPC1 complex, detergent-extracted axonemes were pretreated with HMDEK containing 0.6 M NaCl for 30 min on ice, centrifuged at 14,000 $\times$ *g* for 20 min at 4°C, and then extracted with 0.2 M KI in HMDEK (Mitchell and Sale, 1999). Proteins in the KI extract were further separated by dialysis against HMDEK and centrifugation on a 5–20% sucrose gradient (prepared with HMDEK) at 155,000 $\times$ *g* (35,000 rpm in an SW40 rotor) for 16 h. Gradients were collected from the bottom of the tube in 0.5-ml fractions. Sedimentation standards were outer row dynein (21S) and the CPC1 complex (16S) (internal standards) and were checked against the position of thyroglobulin (19S) and catalase (11S) run in identical gradients.

### *SDS-PAGE and Immunoblotting*

Protein composition was determined by SDS-PAGE on acrylamide gels stained with Coomassie blue. Dynein heavy chains (average $M_r$ = 500,000) and Benchmark Protein Ladder (Life Technologies, Rockville, MD) were used as protein size standards. For immunoblotting, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). Affinity-purified polyclonal rabbit anti-CPC1A (Zhang and Mitchell, 2004) was used at a 1:100 dilution. Rabbit antibodies 16560 and 16562 were raised against peptide sequences TLDAAPGGEHVK and SDGIMVAR, respectively, which correspond to amino acids 269–280 and 301–308 of *C. reinhardtii* PYK4 pyruvate kinase. Peptide synthesis and antibody preparation were provided by Pocono Rabbit Farm and Laboratory (Canadensis, PA). The antibodies were affinity-purified and used at a 1:2000 dilution (0.75 $\mu$g/ml). Mouse monoclonal anti-HSP70 (Affinity Bioreagents, Golden, CO; MA3–006) was used at 1:5000, goat anti-human enolase C-19 (Santa Cruz Biotechnology, Santa Cruz, CA; sc-7455) at 1:200, rabbit antiserum to *C. reinhardtii* chloroplast GAP (Graciet *et al.*, 2004), a gift from Brigitte Gontero, at 1:2000. Rabbit antiserum to spinach chloroplast GAP-A/B (Scheibe *et al.*, 2002) was a gift from Renate Scheibe. Antibodies were detected with peroxidase-labeled goat anti-rabbit, goat anti-mouse (Bio-Rad, Hercules, CA), or bovine anti-goat (Santa Cruz; sc-2350) secondary antibodies developed with ECL reagents (Amersham, Piscataway, NJ). Proteins on blots were detected by staining with amido black or Ponceau-S.

### *Enzyme Assays*

Flagellar fractions were kept on ice until assayed. NP-40 fractions were assayed on the day of preparation; freeze-thaw fractions were assayed within 6 h of thawing. Assay conditions were modified from Burrell (Burrell *et al.*, 1994) with two exceptions. The assay for glyceraldehyde 3-phosphate dehydrogenase in the reverse direction was based on the method of Renate Scheibe (Baalmann *et al.*, 1995). Experiments on enolase activity in sucrose gradient fractions were done using a direct assay that monitored formation of phosphoenolpyruvate as a change in $OD^{240}$ (Spring and Wold, 1975). Final concentrations of components for this assay were as follows: 1 mM 2-phosphoglycerate, 50 mM TRIS, pH 7.5, 15 mM KCl, 5 mM Mg acetate, 1 mM EDTA. Because light scattering by microtubules produces an unacceptably high background at 240 nm, assays on flagellar fractions were done using the Burrell method. Samples (10 $\mu$l) were mixed with enzyme-specific assay

buffer (40 μl), in Greiner 96-well plates (Greiner Bio-One, Frickenhausen, Germany), and assays were initiated by addition of 150 μl of an assay mix containing buffer, substrates, and additional enzymes as noted. Final concentrations of components for each enzyme were as follows: glyceraldehyde 3-phosphate dehydrogenase, forward reaction, 50 mM TRIS, pH 8.0, 20 mM $NaH_2PO_4$, 6 mM cysteine, 1 mM NAD, 1.5 mM glyceraldehyde 3-phosphate; glyceraldehyde 3-phosphate dehydrogenase, reverse reaction, 100 mM TRIS, pH 8.0, 10 mM $MgSO_4$, 1 mM EDTA, 1 mM NADH, 1.5 mM 1,3-bisphosphoglycerate; phosphoglycerate kinase, 100 mM HEPES, pH 7.5, 2 mM Mg acetate, 1 mM EDTA, 1 mM ATP, 0.2 mM NADH, 6.5 mM 3-phosphoglycerate, 3.3 U/ml glyceraldehyde 3-phosphate dehydrogenase; phosphoglycerate mutase, 60 mM HEPES, pH 7.5, 4 mM Mg acetate, 1 mM ADP, 0.15 mM NADH, 3 mM 3-phosphoglycerate, 1 U/ml enolase, 3 U/ml pyruvate kinase, 3 U/ml lactate dehydrogenase; enolase, 100 mM HEPES, pH 7.5, 1 mM Mg acetate, 1 mM ADP, 0.15 mM NADH, 0.5 mM 2-phosphoglycerate, 5 U/ml pyruvate kinase, 3 U/ml lactate dehydrogenase; pyruvate kinase, 50 mM HEPES, pH 7.5, 50 mM KCl, 15 mM $MgCl_2$, 1 mM ADP, 0.15 mM NADH, 5 mM phosphoenol pyruvate, 3 U/ml lactate dehydrogenase. Pyruvate kinase was purchased from Boehringer Mannheim (Indianapolis, IN). All other assay components were from Sigma (St. Louis, MO, except for 1,3-bisphosphoglycerate, which was generated by 30-min incubation at room temperature of 100 mM TRIS, pH 8.0, 10 mM $MgSO_4$, 1 mM EDTA, 35 mM ATP, 70 mM 3-phosphoglycerate, and 3 U/ml phosphoglycerate kinase (Baalmann *et al.*, 1995). Activity was monitored at 25°C by the linear rate of absorbance change at 340 nm, using a Molecular Devices Spectromax385 (Menlo Park, CA). All measurements were averages of duplicate assays run in adjacent wells. Specific activities were determined from assays run at two or three sample concentrations and were limited to results from enzyme concentrations that fell within the linear activity range of each assay. Protein concentration was estimated using a dye binding assay (Bio-Rad, Hercules, CA)) with bovine serum albumin as standard.

### *Proteomics*

For proteomic analysis of subunits of the 16S CPC1-associated complex, a 0.2 M KI extract of axonemes was separated on a sucrose gradient and fractions spanning the 16S region were combined, precipitated with acetone, dissolved in sample buffer, and separated by SDS-PAGE on a 6% gel. The PYK4 pyruvate kinase was fortuitously isolated by immunoprecipitation with a polyclonal antibody directed against the α1 subunit of voltage-gated calcium channels (Chemicon, Temecula, CA; De Jongh *et al.*, 1989). This antibody is directed against a peptide sequence that is highly conserved among different α1 subunits of voltage-gated calcium channels. PYK4 does not contain any peptides that display primary sequence homology to this peptide; apparently, however, the antibody recognizes a conformational epitope in PYK4. Excised gel bands were digested with trypsin and analyzed using MALDI MS and MS/MS by John Leszyk (University of Massachusetts Laboratory for Proteomic Mass Spectroscopy). For PYK4, this analysis led to the identification of four peptide sequences: TLDAAPGGEHVK, SDGIMVAR, GHFEVLER, and VVFHR. To identify additional PYK4 peptides, gel bands were also analyzed by trypsin digestion and ESI-TOF MS/MS by the Australian Proteome Analysis Facility (Macquarie University, Sydney, Australia). This analysis led to the identification of three additional PYK4 peptides: VDIPVLTEKDIDDLQQFAAK, NAEGLVNYDEILR, and PIKDVAAMPK. Proteins are identified by the JGI model name as reported in v2.0 of the *C. reinhardtii* genome database (http://genome.jgi-psf.org/chlamy/).

## RESULTS

### *Enolase Is a Subunit of the CPC1 Complex*

We previously found that *C. reinhardtii* flagella from *cpc1* mutants beat at reduced frequency, but that beat frequency was restored to 90% of wild-type frequency when permeabilized cell models or isolated axonemes were reactivated in the presence of 1 mM ATP (Zhang and Mitchell, 2004), suggesting that this mutation reduces flagellar ATP levels. Although the *CPC1* gene product contains a sequence domain similar to adenylate kinases, we were unable to demonstrate any deficit in adenylate kinase activity in either detergent-permeabilized whole flagella or in the resulting detergent-insoluble axoneme fraction. However, *cpc1* mutant flagella fail to assemble a 16S complex composed of at least four proteins in addition to the CPC1 gene product (Mitchell and Sale, 1999; Zhang and Mitchell, 2004). To determine whether any of these four proteins is directly linked to ATP production, gel bands corresponding to each protein (Figure 2A) were analyzed by mass spectrometry. Peptides identified and the corresponding genes are listed in Table 1.

The highest molecular weight protein in this CPC1 complex (350 kDa) was identified as C_160167 in the *C. reinhardtii* genome database v 2.0 (http://www.genome.jgi-psf.org/chlamy/). Translation of predicted coding regions generates a 2458 amino acid gene product that contains one domain related to adenylate kinases and five domains related to guanylate kinases (Figure 2B). The most N-terminal guanylate kinase-related domain contains most of the highly conserved residues of known enzymatically active guanylate kinases, whereas the remaining four show considerable degeneracy from canonical nucleotide kinase sequences. The second protein in the CPC1 complex (265 kDa) has been previously characterized as the *CPC1* gene product (Zhang and Mitchell, 2004). As noted in Figure 2B, it also has a predicted adenylate kinase domain as the only region with homology to proteins of known enzymatic activity. Orthologues of the CPC1 protein are also present in mammalian cilia from airway epithelia (Ostrowski *et al.*, 1999, 2002). As noted above, assays show no apparent reduction in adenylate kinase activity in mutants lacking this complex (Zhang and Mitchell, 2004), thus adenylate kinase loss would not appear to account for the apparent reduction in ATP concentration in *cpc1* flagella.

The third CPC1 complex protein (135 kDa) was identified as the product of a putative gene (C_340095) with a predicted amino acid sequence (1092 amino acids, 113 kDa) lacking any region of high-sequence similarity to previously characterized proteins. Structural predictions indicate that this protein is mostly alpha helical; database comparisons reveal similarity of two segments to armadillo repeats (Figure 2B).

The CPC1 complex subunit migrating at 79 kDa generated five peptides that all match the sequence of the *C. reinhardtii* HSP70A heat-shock protein (Muller *et al.*, 1992). Further confirmation that HSP70A is a bona fide subunit of the CPC1 complex relied on antibodies previously characterized as specific for HSP70 and that recognize a single 78-kDa flagellar protein in several species, including sea urchin (Stephens and Lemieux, 1999) and *C. reinhardtii* (Bloch and Johnson, 1995). Western blots of sucrose gradient fractions of wild-type central pair extracts show that most of the HSP70A antigen in this fraction cosediments at 16S with the CPC1 proteins (Figure 2C), with a second minor peak having a sedimentation value of about 4S. Furthermore, analysis of axonemes from wild-type, central pair assembly mutant (*pf18*), and *cpc1* cells confirm that HSP70A is reduced in *cpc1* and absent in *pf18* samples (Figure 2D). Immunoblots of gradient fractions show that in *cpc1* extracts all the remaining HSP70 is found in the 4S peak (Figure 2E).

Proteomic analysis of the fifth CPC1 complex subunit (56 kDa) identified eight peptides identical to *C. reinhardtii* enolase, the penultimate enzyme in glycolysis. Western blots using an antibody against a C-terminal peptide of human enolase recognize the 56-kDa CPC1 complex subunit (Figure 2C) and show that this protein is greatly reduced in *cpc1* mutant axonemes and essentially absent in axonemes from *pf18* cells (Figure 2D). However, only a part of the total enolase extracted from axonemes with 0.2 M KI cosediments in a sucrose gradient with other CPC1 complex proteins. The remainder is more broadly distributed, with a peak at a sedimentation value of ~6–7S (Figure 2C). Gradients of *cpc1* extracts indicate that, as with HSP70, any enolase remaining in *cpc1* axonemes is found in this slower-sedimenting peak (Figure 2E).

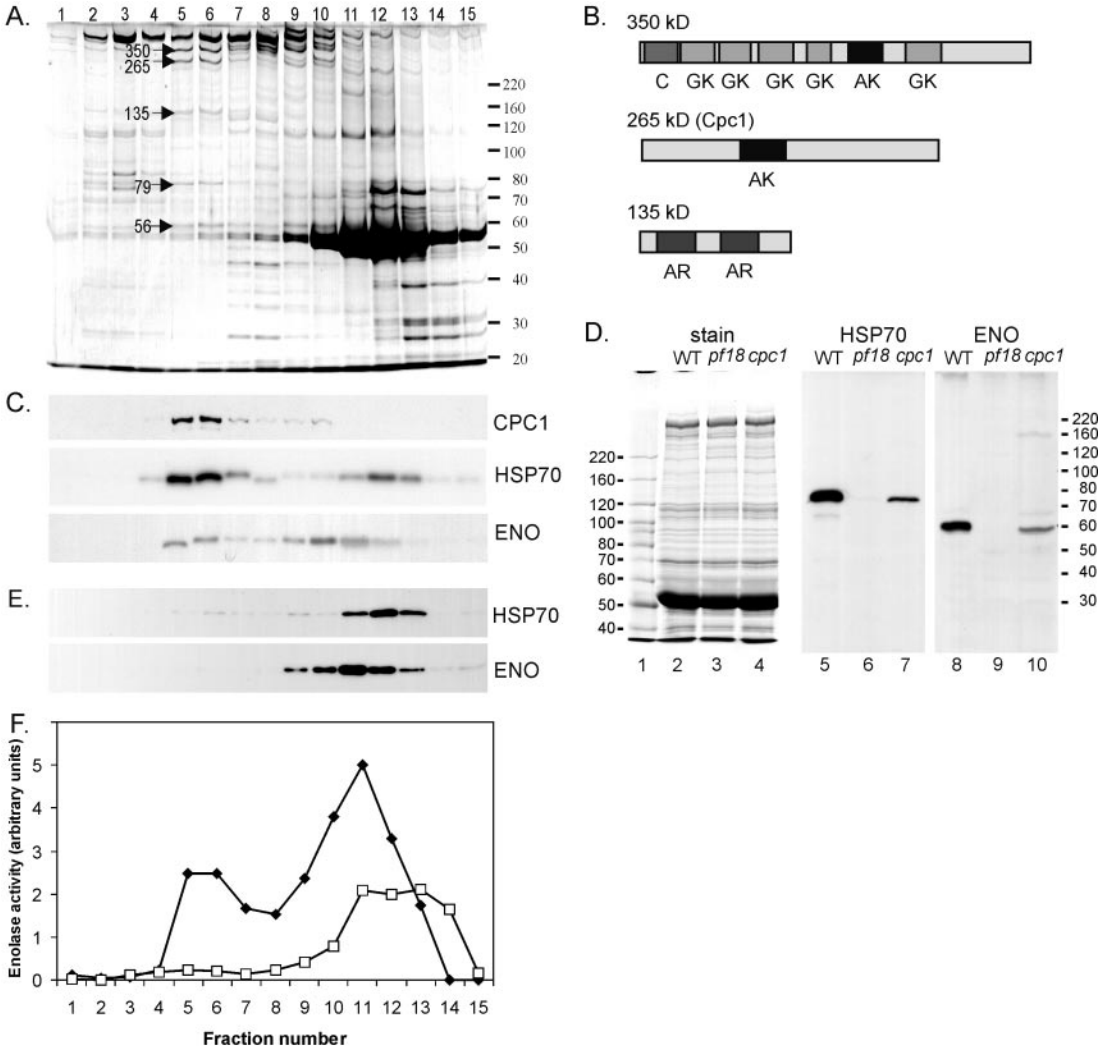


**Figure 2.** Identification of CPC1 complex subunits. CPC1 extracted from wild-type axonemes by 0.2 M KI cosediments on a sucrose gradient with four other proteins. (A) Stained gel of sucrose gradient fractions from wild-type KI extracts with CPC1 complex subunits (arrows) labeled by apparent size. The 265-kDa band was previously determined to be CPC1 (Zhang and Mitchell, 2004). (B) Domain structure of CPC1 and the two novel subunits identified by proteomic analysis (see text and Table 1). The 350-kDa protein contains domains with homology to calpain (C), adenylate kinase (AK), and guanylate kinase (GK); CPC1 contains one AK domain; the 135-kDa protein contains two armadillo repeat domains (AR). (C) Immunoblots of the sucrose gradient fractions shown in A were probed with the indicated antibodies and reveal the cosedimentation of CPC1, HSP70A, and enolase (fractions 5 and 6). Additional peaks of HSP70A and enolase occur higher in the gradient. (D) Gel and corresponding immunoblots of wild-type (WT), central pairless (*pf18*), and *cpc1* axonemal proteins probed with antibodies to HSP70A (HSP70) or enolase (ENO). Both proteins are missing from *pf18* axonemes and greatly reduced in *cpc1* axonemes. Gels were 7% acrylamide for A and C and the stained image in D and 10% acrylamide for the immunoblots in D. (E) Immunoblots of sucrose gradient fractions of *cpc1* KI extracts were probed with the indicated antibodies and reveal the loss of enolase and HSP70A in fractions 5 and 6. The sizes (in kDa) of molecular mass standards are indicated next to each gel (A and D) and the enolase blot (D). The gel in A and the anti-CPC1 immunoblot in C appeared in a previous publication (Zhang and Mitchell, 2004). (F) Enolase activity in axonemal extracts. Enolase activity was measured in sucrose gradient fractions, similar to those shown in A, prepared with extracts from equal numbers of wild-type (●) or *cpc1* (○) axonemes. Data are representative of two independent experiments.

As further confirmation that the 56-kDa protein retained other expected properties of enolase, enzyme assays were performed on sucrose gradient fractions of KI extracts from both wild-type and *cpc1* mutant axonemes. As illustrated in Figure 2F, enolase activity occurs in two peaks in the wild-type gradient that coincide with peaks identified antigenically to contain enolase (Figure 2C). The *cpc1* gradient lacks enolase activity corresponding to the wild-type 16S sedimentation peak and has greatly reduced activity associated with the 6–7S peak, which suggests that at least part of the wild-type 6–7S peak represents enolase that has dissociated from the CPC1 complex. Thus both immunoblot and enzyme activity data indicate that much of the enolase in wild-type axonemes is associated with the CPC1 complex (Figure 2, C, E, and F).

To determine the proportion of total flagellar enolase that is associated with the axoneme, immunoblots of whole flagella, and of separated axonemal and membrane + matrix (detergent soluble) flagellar fractions from wild-type, *cpc1*, and *pf18* cells were probed for enolase (Figure 3). After treatment with NP-40 detergent, more than half of the enolase present in wild-type flagella appears in the detergent

**Table 1.** Proteomic identification of CPC1 complex subunits

| Subunit size (kDa) | Gene[a] | Product | Peptides |
|---|---|---|---|
| 350 | C_160167 | Unnamed | VLPAGGKPGSGPVLLSAEQLAER |
| | | | AAAVPPPTPPGPEPSAPSASHGK |
| | | | VIKDGSTLVLDPTAQLR |
| | | | LASVGAALQASKGER |
| | | | LRTLMANAGLLELPR |
| | | | VVYLAVDVAEQDVR |
| | | | LRDHLTETDEEIAAR |
| | | | EAASKRHELASQVK |
| | | | GGAWGGAGRPALA |
| | | | TGALPEGAEPVKLR |
| | | | LDSFVEAYRPSNR |
| | | | ASGVARPVPSNR |
| | | | QANNFAEWR |
| 135 | C_340095 | Unnamed | ASSPGGASALDEFGEEPDIRPLR |
| | | | AIQAQFAESGVKPTAPDR |
| | | | LGSPGGGAGIGSIPVSR |
| | | | VLLLYVSPGEPHPAVR |
| | | | SALADREPPTSPSAR |
| | | | LHTLSPLCPEEYER |
| | | | GGAPDPAEQAR |
| | | | RWNSDQLATLR |
| 79 | C_1340012 (P25840) | HSP70A | AVVTVPAYFNDSQR |
| | | | TTPSYVAFTDTER |
| | | | DAGMIAGLEVLR |
| | | | NSLENYAYNMR |
| | | | LVNHFANEFQR |
| 56 | C_160138 (P13683) | Enolase | KGMFRAAVPSGASTGVHEAVELR |
| | | | LTTENICQVVGDDILVTNPVR |
| | | | IEEELGENAVYAGESWR |
| | | | ALGOLTPPEIVKVVGR |
| | | | VNQIGTITESIEAVR |
| | | | MKDLDGTDNKGK |
| | | | GNPTVEADVYTR |
| | | | EAGWGVMTSHR |

[a] Gene names are those designated by the JGI to label gene models in version 2.0 of the *Chlamydomonas* genome database (http://genome.jgi-psf.org/chlamy/). GenBank accession numbers, when available, are shown in parentheses.

soluble (Figure 3B, lanes M+M), rather than the axonemal (Figure 3B, lanes AXO) fraction. The amount of enolase protein is reduced in total flagella and axonemes from both *cpc1* and *pf18* cells (Figure 3B), consistent with the results shown in Figure 2D. Comparison of *pf18* with other central pair assembly mutants (*pf15, pf19, pf20*) shows that flagellar enolase is similarly detergent extractable in all of these mutants (unpublished data), thus the majority of flagellar enolase is in the detergent-soluble compartment. In *cpc1* and *pf18* flagella the *axonemal* enolase is reduced or missing, respectively, indicating that all of the enolase that is structurally anchored to the axoneme is present in the central pair complex.

### *Glycolytic Enzyme Activity in Chlamydomonas Flagella*

ATP formation from the breakdown of glucose has been conceptually divided into two parts, a first half that requires phosphorylation by ATP and a second half (Figure 4) that generates excess ATP. The two ATP generating steps of glycolysis are thought to take place in the cytoplasm in *C. reinhardtii*, whereas earlier steps in glucose degradation occur in the chloroplast (Klein, 1986). Because enolase does not

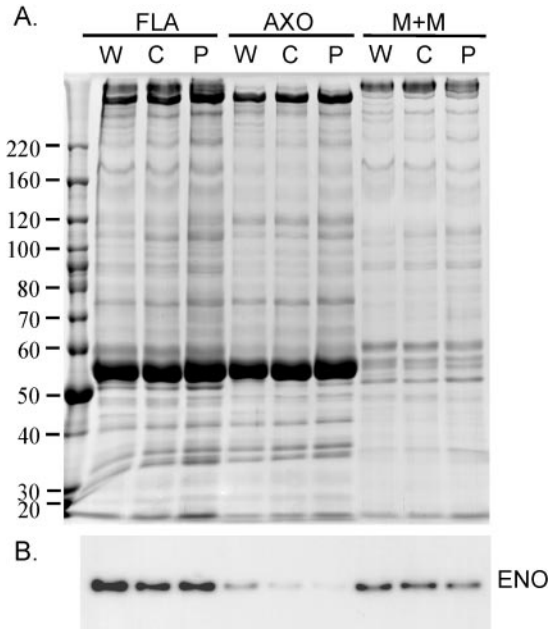


**Figure 3.** Blot analysis of flagellar enolase in wild-type and central pair assembly defective mutants. (A) Stained gel of whole flagella (FLA), and the insoluble axoneme (AXO) and soluble membrane + matrix (M+M) fractions generated by treatment with NP-40 detergent. Samples were prepared from wild-type (W), *cpc1* (C), or *pf18* (P) cells. (B) Immunoblot of a gel identical to A except that sample loads were reduced 50-fold and the separating gel contained 1 M urea, probed with anti-enolase (C-19). The sizes (in kDa) of molecular mass standards are indicated next to the stained panel.

directly catalyze ATP synthesis, we looked for additional glycolytic enzymes in *C. reinhardtii* flagella and flagellar fractions. For these experiments, flagella were fractionated using two different methods. Treatment with the nonionic

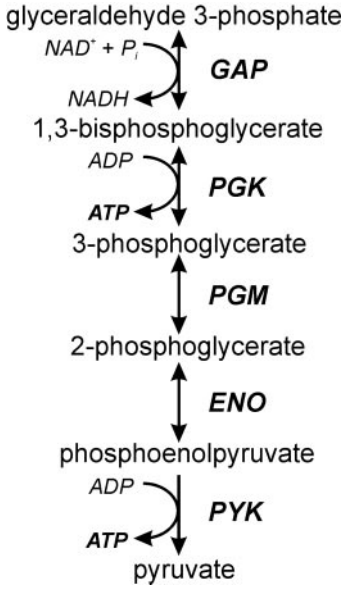


**Figure 4.** Diagram summarizing steps of glycolysis known to occur in the cytosol in *Chlamydomonas,* including the two steps that can contribute to ATP synthesis.

**Table 2.** Activity of glycolytic enzymes in flagella

Specific activity (nmol/mg/min)

| | Cytoplasmic extract | NP-40-treated flagella | | Freeze-thaw-treated flagella | |
|---|---|---|---|---|---|
| Enzyme[a] | Total | Total | % Soluble | Total | % Soluble |
| GAP | 508 | 0 | — | 0 | — |
| PGK | 500 | 0 | — | 0 | — |
| PGM | 1004 | 590 | 94 | 379 | 89 |
| ENO | 77 | 68 | 47 | 61 | 52 |
| PYK | 102 | 136 | 76 | 140 | 23 |

[a] GAP, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; ENO, enolase; PYK, pyruvate kinase.

detergent NP-40 was used to simultaneously solubilize the bulk of the membrane proteins and release detergent-soluble (matrix) proteins, whereas axonemal microtubules and any microtubule-associated proteins remained in the NP-40-insoluble fraction. Alternatively, freeze-thaw treatment was used to release only matrix proteins (Zhang and Snell, 1995; Cole *et al.*, 1998), whereas the major membrane glycoprotein (Bloodgood and Salomonsky, 1994) remained with axonemes in the insoluble fraction. Activity was measured for each of the five enzymes of the lower half of glycolysis (Figure 4) beginning with glyceraldehyde 3-phosphate dehydrogenase (GAP). Activities were measured on at least three independent flagellar preparations for each enzyme, with similar results. The results from one preparation are summarized in Table 2. Interestingly, easily measurable activity for only three of these five enzymes was detected in whole flagella. We were unable to detect GAP activity in flagella using either 1,3-bisphosphoglycerate and NADH (or NADPH) as substrates (reverse reaction direction), or using $P_i$, glyceraldehyde 3-phosphate, and NAD (or NADP) as substrates (forward reaction direction), under conditions that readily detected the GAP activity of whole cell extracts (Table 2 and unpublished data). Phosphoglycerate kinase (PGK) activity was also readily detected in cytoplasmic extracts, but not in flagella or flagellar extracts. Activities for the last three enzymes in the pathway, phosphoglycerate mutase, enolase, and pyruvate kinase were all detected in flagella (Table 2) and displayed similar activity levels following either NP-40 treatment or freeze-thaw treatment. Collectively, these enzymes could support the second of the two ATP-generating steps of glycolysis, indicating that *C. reinhardtii* flagella have the capacity to produce one ATP molecule per substrate molecule (i.e., 3-phosphoglycerate) consumed.

After detergent treatment, most of the pyruvate kinase and phosphoglycerate mutase activities were released into the soluble fraction, whereas about half of the enolase activity remained with axonemal microtubules (Table 2). This result for enolase activity differs from the results of immunoblots probed with anti-enolase antibodies, described above, in which much less than half of the enolase remained associated with axonemal microtubules (Figure 3). The detergent-soluble fraction of enolase does not appear to be membrane-associated, as freeze-thaw and NP-40 treatments produced similar levels of soluble enzyme activity. Half of the enolase activity in NP-40-treated flagella is therefore generated by much less than half of the enolase protein, which suggests that axoneme-bound enolase has a higher specific activity than the enolase that is released by NP-40 treatment. Most of the flagellar phosphoglycerate mutase activity was released into a soluble form by either NP-40 or freeze-thaw treatment, indicating that this enzyme resides in the flagellar matrix compartment. In contrast, only 23% of pyruvate kinase activity was soluble after freeze-thaw treatment of flagella compared with 76% after detergent treatment, suggesting some form of membrane association for this enzyme. Although we cannot rule out the possibility that these three enzymes may physically interact in vivo, their unique fractionation patterns argue against their association into a complex or onto a common scaffold.

### *Identification of Flagellar Enzyme Isoforms*

Queries of the *C. reinhardtii* genome sequence database (http://genome.jgi-psf.org/chlamy/), indicated that several glycolytic enzymes are represented by multiple isoforms. As a first step toward understanding how glycolytic enzymes could be targeted to the flagellar compartment, we sought to determine which isoform of each enzyme is present in flagella. The *C. reinhardtii* genome encodes two phosphoglycerate mutase isoforms, one similar to the cofactor-dependent forms common in metazoans (C_340113) and one similar to the cofactor-independent forms common in higher plants (C_270009). As only the cofactor-dependent forms are inhibited by vanadate (Carreras *et al.*, 1980), we tested the effects of vanadate on flagellar phosphoglycerate mutase activity. Total flagellar phosphoglycerate mutase activity was inhibited by 78% in 1 $\mu$M vanadate and 95% in 10 $\mu$M vanadate, indicating that the cofactor-dependent gene product (C_340113) predominates in the flagellar compartment.

Previous studies have concluded that enolase is present in cytoplasmic but not chloroplast fractions of *C. reinhardtii* cells (Klein, 1986; Klock and Kreuzberg, 1991). As there is only a single isoform of enolase in the *C. reinhardtii* genome, the same isoform must be used in both cytoplasm and flagella.

Pyruvate kinase activity is also primarily cytoplasmic rather than chloroplastic in *C. reinhardtii* (Klein, 1986). As there are five putative pyruvate kinase sequences in the *C. reinhardtii* genome, individual isoforms may be compartment-specific. A flagellar isozyme of pyruvate kinase was initially identified through proteomic analysis of a high-molecular-mass protein that copurified with flagellar membrane/matrix proteins and cross- reacted with an antibody against voltage-gated calcium channels (see *Materials and Methods*). The predicted sequence of this unusual pyruvate kinase (termed PYK4) is shown in Figure 5A, and the eight peptides identified by mass spectrometry are underlined. PYK4 contains four domains with pyruvate kinase homology and has a predicted molecular mass of 230 kDa (Figure 5B). Antibodies raised against two peptides of this gene product (bolded in Figure 5A) show specificity toward a single 240-kDa band in both whole cells (unpublished data) and flagella (Figure 5C). One of these antibodies was used to compare activity levels with protein levels in flagellar fractions. As illustrated in Figure 5D, PYK4 follows a fractionation pattern, after either NP-40 or freeze-thaw treatment, similar to the fractionation of pyruvate kinase activity reported in Table 2. Determination of the distribution of PYK4 along the flagellum awaits identification of conditions that permit accessibility of these epitopes in material preserved for immunofluorescence. We conclude that *C. reinhardtii* flagella likely contain only one isoform each of phosphoglyc-

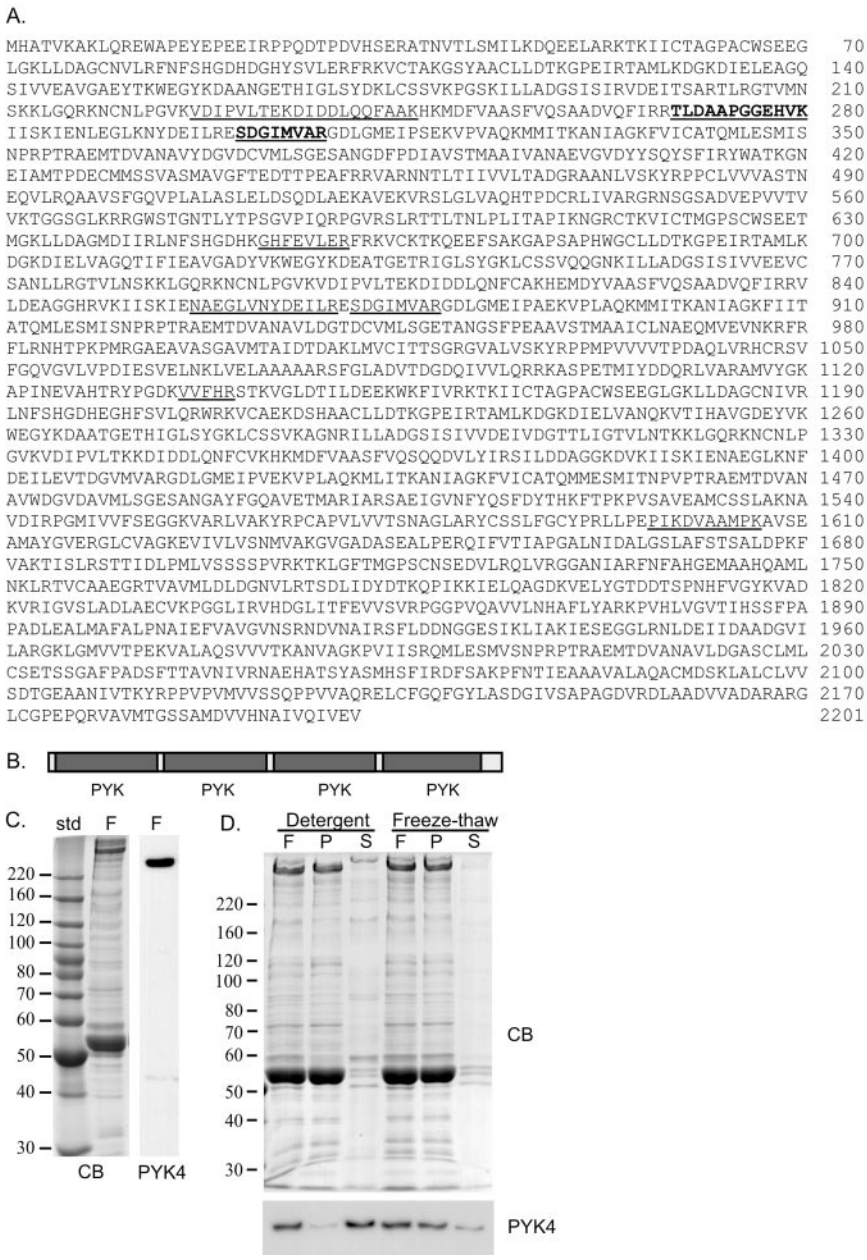


**Figure 5.** Identification of pyruvate kinase in flagellar fractions. (A) Sequence of the pyruvate kinase encoded by C_2590004, identified by proteomic analysis of flagellar extracts. Peptides identified by mass spectroscopy are underlined. Two of these (bold) were used to raise antibodies. (B) The C_2590004 gene product, PYK4, consists of four pyruvate kinase domains (PYK) separated by short spacers. (C) Gel stained with Coomassie blue (CB) and immunoblot (PYK4) of flagellar proteins (lanes F) showing the specificity of anti-PYK4 antibody 16562 for a 240-kDa band. (D) Gel (CB) and immunoblot (PYK4) of flagellar fractions probed with anti-PYK4 antibody 16562. Flagella (F) were fractionated into insoluble axonemal pellets (P) and soluble supernates (S) by either detergent or freeze-thaw treatments. The sizes (in kDa) of molecular mass standards (std) are indicated next to the stained panels.

erate mutase (cofactor-dependent isoform; C_340113), enolase (C_160138), and pyruvate kinase (PYK4; C_2590004).

## DISCUSSION

### *Metabolic Compartmentalization of Cilia and Flagella*

Metabolic enzymes may be sequestered in subcellular compartments or anchored to specific structures to provide localized regulation of metabolite concentration, just as signaling enzymes are organized into cascades through their association with scaffold proteins. The high ATP hydrolysis rates required for skeletal muscle contraction are supported by the anchoring to sarcomeres of glycolytic enzymes, including GAP, phosphoglycerate kinase, phosphoglycerate mutase, and enolase (Foucault *et al.,* 1999; Sullivan *et al.,* 2003). Glycolysis is also an important source of energy for sperm motility in mammals (Mukai and Okuno, 2004) and some enzymes such as GAP (Westhoff and Kamp, 1997) and enolase (Gitlits *et al.,* 2000) appear to be structurally anchored in sperm. In a similar way, as shown here, the high ATP turnover rates needed in *C. reinhardtii* for flagellar motility are also supported by glycolytic enzymes targeted to the flagellar compartment. The first two enzymes in the ATP-generating part of the pathway, GAP and phosphoglycerate kinase, could not be detected in *C. reinhardtii* flagella, even though they were easily measured in cytoplasmic extracts. However, each of the enzymes needed to complete the second of the two ATP-generating steps is present in flagella, as seen by direct activity measurements and, in two cases, by proteomic analysis and immunoblotting with specific antibodies. This limitation of glycolysis in flagella to the final three steps makes sense in light of previous work showing that in *C. reinhardtii*, phosphoglyerate mutase, enolase, and pyruvate kinase are localized exclusively in the

cytoplasm, rather than the chloroplast, whereas earlier glycolytic enzymes, through aldolase, are localized primarily in the chloroplast (Klein, 1986; Klock and Kreuzberg, 1991). A similar compartmentation is seen in trypanosomes, where glycolytic enzymes before phosphoglyerate mutase are sequestered in a modified peroxisome known as the glycosome (Kessler and Parsons, 2005). It is curious, given the evidence presented here, as well as earlier work, that recent proteomics suggest the presence of additional glycolytic enzymes in the *C. reinhardtii* flagellum (Pazour *et al.*, 2005). This leaves open the possibility that additional glycolytic activities may be revealed in *C. reinhardtii* flagella through the use of alternative assay procedures.

### *Flagellar-specific Glycolytic Enzymes*

If the same glycolytic enzymes are targeted to both cytoplasmic and flagellar compartments, then flagellar localization may depend on an interaction of each glycolytic enzyme with a protein or mechanism that targets proteins to the flagellum. As the *C. reinhardtii* genome has only one enolase gene, the same protein must serve for both cytoplasmic and flagellar glycolysis. Linkage to central pair proteins such as the CPC1 complex may be important for anchoring enolase in the flagellar compartment, but is not essential for targeting to this organelle, as some enolase remains present in whole *cpc1* flagella, which lack this complex.

Phosphoglycerate mutases occur in two forms, 2,3-bisphosphoglycerate-dependent or -independent, and one copy of each form is encoded in the *C. reinhardtii* genome. We observed nearly complete inhibition of phosphoglycerate mutase activity in the flagellar fraction by low concentrations of vanadate, indicating that a cofactor-dependent isoform is present in flagella. It is unclear at present if this isoform is also used in the cytoplasm or chloroplast and what mechanisms target this protein to the flagellum.

The pyruvate kinase isoform (PYK4) that we identify in flagella is unusual. Most known pyruvate kinases function as tetramers of four identical ~50-kDa subunits (Munoz and Ponce, 2003), suggesting that PYK4 exists as a monomer containing four functional pyruvate kinase domains. A similar four-domain pyruvate kinase was characterized in another alga as a chloroplast enzyme (Knowles *et al.*, 2005), but because there are three typical single-domain pyruvate kinase isoform genes and two four-domain pyruvate kinase genes in *C. reinhardtii*, the phylogeny and subcellular locations of these isoforms require further analysis.

### *Functions of Glycolytic Enzymes in the Flagellum*

Flagellar motility depends on dynein ATPases that are distributed along the entire flagellum except the extreme distal tip, where B-tubules stop (approximately the last 0.2 $\mu$m of the 12-$\mu$m *C. reinhardtii* flagella). That flagellar beat frequency varies with ATP concentration has been demonstrated in numerous systems (Brokaw, 1967), including *C. reinhardtii* (Zhang and Mitchell, 2004). We found that reduced amounts of flagellar enolase in the *cpc1* mutant, which should lead to reductions in ATP production by the pyruvate kinase-dependent step of glycolysis, do correlate with significantly reduced intraflagellar ATP concentrations as monitored by in vivo beat frequency (Zhang and Mitchell, 2004). This suggests that ATP generated by glycolysis in situ, although perhaps not the sole source of flagellar ATP, is important for flagellar motility. ATP generated by glycolysis likely supplements the ATP provided directly through diffusion from the cytoplasm, but the relative contributions of these two sources remain to be determined. The diffusion of small metabolites from the cytoplasm into the flagellar compartment is presumed to be limited by both the size of the opening and the presence of structures within the flagellar transition zone. The independent diffusion of two different small compounds, ATP and 3-phosphoglycerate, may ameliorate the effects of this diffusion barrier, providing two different energy sources for the flagellum. The characterization of enolase as a subunit of the CPC1 complex indicates that the anchored (axonemal) fraction of this enzyme is distributed along the length of the flagellum; this in turn suggests that ATP production may be similarly distributed along the organelle.

In addition to powering beating motility, ATP is also essential as a substrate for IFT and for protein kinases, which are important regulators of ciliary and flagellar motility (Porter and Sale, 2000). Recent evidence suggests that one function of glycolysis in sperm flagella is production of ATP for use by tyrosine kinases implicated in capacitation (Travis *et al.*, 2001).

Although the ATP concentration dependence of flagellar kinases and IFT motors have not been determined in *C. reinhardtii*, the apparent reduction in ATP concentration in *cpc1* flagella has no discernable effect on flagellar length (an IFT-dependent value) or kinase-regulated tactic motility responses (Mitchell and Sale, 1999; Zhang and Mitchell, 2004).

### *Composition of the CPC1 Complex and C1b Projection*

Our proteomic analysis of proteins associated with CPC1 identified two previously uncharacterized proteins of 350 and 135 kDa, and two proteins not previously known to be associated with the central pair, HSP70A and enolase. The 135-kDa protein has no close homologues and, based on secondary structure predictions, is likely to perform a structural role. The highest molecular weight (350 kDa) member of the CPC1 complex has several domains with homology to guanylate kinases, and one domain homologous to adenylate kinases. Although it is likely that flagellar adenylate kinases play a role in maintaining ATP concentrations, based on our previous work (Zhang and Mitchell, 2004), neither CPC1 nor the 350-kDa protein contribute measurably to total axonemal adenylate kinase activity under standard in vitro assay conditions. In trypanosomes, a >80% reduction in flagellar adenylate kinase activity has no detectable effect on flagellar beat activity (Pullen *et al.*, 2004), suggesting that flagellar isoforms of this enzyme may not be a major determinant of organellar ATP concentrations. Guanylate kinase activity has no known role in central pair function, but maintenance of flagellar GTP concentrations may be essential for both guanylate cyclase activity and tubulin polymerization. A nucleoside diphosphate kinase that may also contribute to maintenance of GTP concentrations has been localized to *C. reinhardtii* radial spokes (Patel-King *et al.*, 2004). Alternatively, large flagellar structures such as the C1b projection may be formed in part by proteins that have evolved through the accumulation of preexisting structural domains, whose former enzymatic functions are no longer required.

HSP70A could play a chaperone role in assembly of cilia and flagella, and has previously been shown by immunolocalization to be concentrated near the tips of *C. reinhardtii* flagella (Bloch and Johnson, 1995). Here we show that HSP70A is also associated with the CPC1 protein in the central pair C1b projection. Because chaperones of the HSP70 family are ATPases that change conformation and affinity for binding partners as a result of ATP hydrolysis (Young *et al.*, 2003), this protein could potentially act as a conformational switch for central pair regulation of flagellar motility. Although HSP70 chaperones often function in con-

junction with HSP40 or HSP90 family members, the only known HSP40 protein identified in *C. reinhardtii* flagella resides near the base of the radial spoke (Yang *et al.*, 2005) and is thus unlikely to interact with CPC1-associated HSP70A. HSP90 homologues have also been identified in cilia and flagella from divergent phyla (Williams and Nelsen, 1997; Stephens and Lemieux, 1999) and have been linked to spermatogenesis (Yue *et al.*, 1999) and central pair assembly (Zhang *et al.*, 2004) in *Drosophila*, but have not been specifically localized to the central pair apparatus.

In summary, in situ glycolytic synthesis of ATP is needed for normal rates of flagellar motility in *C. reinhardtii*. Unlike mammalian sperm flagella, the flagella in *C. reinhardtii* retain only one of the two ATP-generating steps of glycolysis and thus depend on diffusion or transport of 3-phosphoglycerate from the cytoplasm. Although two of the enzymes in this pathway (phosphoglycerate mutase and pyruvate kinase) may be uniquely targeted to flagella, the third (enolase) must retain targeting signals for both cytoplasmic and flagellar compartments, which may account for the unique association of enolase with a central pair microtubule complex.

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