Phosphorylation in Isolated *Chlamydomonas* Axonemes: A Phosphoprotein May Mediate the Ca^{2+}-dependent Photophobic Response

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ABSTRACT An in vitro system was devised for studying phosphorylation of *Chlamydomonas reinhardtii* axonemal proteins. Many of the polypeptides phosphorylated in this system could be identified as previously described axonemal components that are phosphorylated in vivo. The in vitro system apparently preserved the activities of diverse axonemal kinases without greatly altering the substrate specificity of the enzymes. The in vitro system was used to study the effect of calcium concentration on axonemal protein phosphorylation. Calcium has previously been demonstrated to initiate the axonemal reversal reaction of the photophobic response; the in vitro system made it possible to investigate the possibility that this calcium effect is mediated by protein phosphorylation. Calcium specifically altered the phosphorylation of only two axonemal proteins; the phosphorylation of an otherwise unidentified 85,000 M_r protein was repressed by calcium concentrations $\geq 10^{-6}$ M, while the phosphorylation of the previously identified 95,000 M_r protein b4 was stimulated by calcium at concentrations $>10^{-6}$ M. Protein b4 is one of six polypeptides that are deficient in the *mbo* mutants, strains that do not exhibit a photophobic reversal reaction. Therefore, this calcium-stimulated phosphorylation may be involved in initiating the photophobic response. Neither calmodulin nor the C-kinase could be implicated in b4 phosphorylation. The calcium-dependent activation of the b4 kinase was not affected by several drugs that bind to and inhibit calmodulin, or by the addition of exogenous calmodulin. Activators and inhibitors of the calcium-phospholipid-dependent C kinase also had no effect on b4 phosphorylation.

The unicellular, biflagellate alga *Chlamydomonas reinhardtii* displays a characteristic avoiding reaction in response to changes in light intensity. This photophobic response involves a transient alteration in the flagellar waveform. During the avoiding reaction, the beat pattern switches from the predominant asymmetric high amplitude stroke to a symmetrical stroke (45). When the two flagella, located on the anterior pole of the cell, use the asymmetric stroke, the cell moves in a forward direction, whereas when the flagella use the symmetrical stroke the cell reverses the direction of movement. The photophobic response has been shown to require the presence of $>10^{-4}$ M calcium ions in the surrounding medium (47). Studies on functional axonemes, reactivated in vitro by the addition of ATP, have established that the calcium acts directly on the axoneme to induce the change in the waveform (3, 18, 19).

One approach to studying the calcium control of the waveform has been to isolate and analyze mutants that do not display the normal photophobic response. Nakamura originally isolated two strains that show a persistent flagellar-type stroke and do not display a photophobic response (32, 33). We have isolated several additional mutants with this phenotype; we have designated the loci involved *mbo1*, *mbo2*, and *mbo3* for moves backward only (49). In vitro the reactivated axonemes of such mutants do not show a calcium-induced alteration in waveform, and ultrastructural and biochemical analyses of the mutant axonemes have revealed a consistent pattern of defects associated with the *mbo* phenotype (49). The *mbo* mutants show specific deficiencies in the doublet specializations located in the proximal portion of the axoneme, which are associated with the lateral doublets 5 and 6 and the medial doublet 1. In wild-type cells these speciali-

THE JOURNAL OF CELL BIOLOGY · VOLUME 101 NOVEMBER 1985 1702-1712

zations include small, triangular structures that are within the lumina of the B-subfibers of these three doublets (15). The intraluminal structures were described by Hoops and Witman as beak-like projections (15). The *mbo* mutants retain the doublet 1 specialization, but are missing the projections of doublets 5 and 6 (49). All the *mbo* mutants examined also show a defined biochemical lesion; six polypeptides are consistently deficient in all *mbo* mutants. Some or all of these proteins apparently form the B-subfiber projections (49; unpublished observations). Four of these six components are phosphoproteins that, in wild-type axonemes, are highly labeled after a short pulse with ^{32}P-phosphate. Therefore, there appears to be a rapid turnover of these phosphate groups in assembled flagella.

We examined the possibility that the phosphorylation of these polypeptides might be important in the photophobic response. For example, the calcium-mediated control of the beat pattern might be executed by specific phosphorylation reactions. In many systems calcium initiates a cellular response by specifically activating kinases or phosphatases (see reviews in references 7, 24, and 35). The availability of methods for preparing and isolating functional axonemes that retained the ability to recognize changes in calcium concentration and to respond appropriately (3) allowed us to carry out in vitro phosphorylation studies and to study the effects of calcium on protein phosphorylation. Calcium specifically altered the phosphorylation of only two axonemal proteins; one of these was protein b4, a phosphoprotein that is deficient in the *mbo* mutants. This calcium-stimulated phosphorylation may be involved in initiating the photophobic response.

MATERIALS AND METHODS

Human erythrocyte calmodulin was obtained from Calbiochem-Behring Corp. (La Jolla, CA), sodium vanadate was purchased from Fisher Scientific Co. (Pittsburgh, PA), the drug trifluoperazine was obtained from SmithKline Diagnostics Inc. (Sunnyvale, CA), W-7 and W-5 were obtained from Seikagaku Kogyo Co., while the 2-norchlorpromazine was a gift from Milton Cormier (University of Georgia), and the calmidazolium was purchased from Boehringer Mannheim Diagnostics, Inc. (Houston, TX). The phosphoamino acid standards and L-phosphatidyl serine were obtained from Sigma Chemical Co. (St. Louis, MO), as were the dibutyryl cyclic nucleotides, the inhibitor of the cAMP-dependent kinase, and caffeine. The γ-^{32}P-ATP and the adenosine 5′(thio)triphosphate(^{35}S), (ATPγS),[1] were obtained from New England Nuclear (Boston, MA). The myosin light chain kinase substrate peptide KM14SM and inhibitor KM14A3 were gifts from Dr. M. Watterson, Vanderbilt University. The following procedures have been previously described: culture of the cells (29), one- and two-dimensional gel electrophoresis (39–41), and pulse-labeling of cells with ^{32}P-phosphate (49). Protein concentration was determined by the method of Lowry (27). Polyacrylamide gels were visualized by silver staining as described by Merrill et al. (31).

Preparation of Axonemes

The axonemes for the standard in vitro phosphorylation were prepared by a modification of the procedure of Bessen, Fay, and Witman (3). Cells were resuspended in M-N/5 (minimal medium minus nitrogen), washed twice in the same medium, then twice in 10 mM HEPES pH 7.4. The cells were resuspended in 10 mM HEPES, 5 mM $MgCl_2$, 1 mM dithiothreitol pH 7.4 at ~4 × 10^8 cells/ml, then deflagellated by the addition of 25 mM dibucaine. During the 3-min deflagellation, cells were mixed by rapidly pipetting the suspension. After 3 min, EGTA was added to the cell suspension to a final concentration of 0.5 mM, then the suspension was diluted with an equal volume of the deflagellation buffer. The flagella were separated from the cell bodies by three cycles of differential centrifugation. The supernatant, containing the flagella, was diluted with an equal volume of solution A: 50 mM Na-HEPES, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 50 mM KCl, pH 7.4, and Nonidet P-40 was added to a final concentration of 0.1%. The axonemes were pelleted by a 30-min spin at 34,000 *g*. The pellets were gently resuspended in and washed with solution B, which contains 30 mM HEPES, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EDTA, 25 mM KCl pH 7.4, then once again pelleted by the same centrifugation. The axonemes were again resuspended in the same solution at a concentration of 2–10 mg/ml. No solutions were allowed to come into contact with a pH electrode. These axonemes could not be reactivated by addition of ATP. However, if 1% and 0.5% polyethylene glycol were included in solutions A and B, respectively, 0–40% of the axonemes were motile on addition of ATP.

[1] *Abbreviations used in this paper:* ATPγS, adenosine 5′(thio)triphosphate(^{35}S).

Phosphorylation Reaction

The standard in vitro phosphorylation assay was carried out by adding 20 μl of the axonemal suspension to 25 μl of the solution B, containing 0.1 mM ^{32}P-ATP (10,000–15,000 cpm/pmol), 0.3 mM sodium vanadate, buffered with $CaCl_2$-EGTA to obtain desired concentrations of free calcium ions calculated as described by Katz et al. (21). Potential activators or inhibitors were added to the ATP solution. After a 5-min incubation at room temperature, the phosphorylation reaction was ended by adding 5 μl of a 5% SDS solubilization solution (39) and boiling the sample for 1 min. To estimate incorporation of counts into protein, 1 μl of the 50-μl sample was spotted onto a Whatman filter, washed three times with 10% trichloroacetic acid solution, then dried and counted. The samples were prepared and run on the two-dimensional gel electrophoresis system as previously described (39–41). Protein thiophosphorylation was carried out in vitro by the same procedure, substituting 6 μM ATPγS (11,000 cpm/pmol) for ^{32}P-ATP and eliminating vanadate from the reaction mixture. In vitro phosphorylation assays using flagella or axonemes prepared by the usual pH shock deflagellation (9) were done in 10 mM HEPES pH 6.8–8.3, 0–1% Nonidet P-40, 0–100 mM NaF, 0–100 mM NaCl, 5 mM $MgCl_2$, then run on one- and two-dimensional gels as usual. ^{32}P-labeled proteins and ^{35}S-thiophosphorylated proteins were visualized by autoradiography with Kodak SB-5 film at −70°C with an intensifying screen for 10 d, and at room temperature for 70 d, respectively.

Phosphoamino Acid Analysis

Phosphoamino acid analysis was carried out essentially as described by Hunter and Sefton (17). For analysis of the total axonemal proteins, the 15% trichloroacetic acid precipitate of in vivo or in vitro phosphorylated axonemes was prepared and then extracted with 2:1 chloroform/methanol. The protein was resuspended in 6 M HCl, sealed within a micropipette, and hydrolyzed at 100°C for 2 h. The hydrolyzed samples were diluted in water and lyophilized several times. The final samples were suspended in a solution containing 1.5 mg/ml each of phosphoserine, phosphothreonine, and phosphotyrosine, and spotted on thin layer chromatography plates (0.1-mm cellulose MN-300 precoated glass plates from Brinkmann Instruments Co., Westbury, NY). The first dimension electrophoresis was in a pH 1.9 buffer (78 acetic acid/25 formic acid/897 water) at 1 kV for 1½ h. The second dimension was done at 0.75 kV for 1 h in a pH 3.5 buffer (50 acetic acid/5 pyridine/945 water). The plates were then stained with ninhydrin and exposed to Cronex film for 3 d at −70°C with an intensifying screen. For quantitation, the ninhydrin-stained spots were scraped from the glass backing, and the radioactivity in each was counted.

For analysis of individual components, unfixed two-dimensional gels were dried and exposed to Cronex film overnight. The relevant spots were cut out and rehydrated in 25 mM $KHCO_3$ pH 7. The gels were then cut into small pieces and incubated in the buffer with 0.1 mg/ml proteinase K overnight at 42°C. The extraction was repeated, and the gels were then washed several times with the $KHCO_3$ buffer alone. The pooled extracts were lyophilized, then resuspended in water and lyophilized again. This was repeated several times, then the sample was resuspended in 6 M HCl, hydrolyzed, and analyzed in the same way as the total protein samples.

RESULTS

In Vitro Phosphorylation System

To study the possible involvement of a calcium-dependent kinase or phosphatase in the photophobic response, an axonemal in vitro phosphorylation system was established using purified axonemes, able to respond to calcium by altering the waveform. A protocol that yields reactivatable axonemes was initially used to prepare samples for in vitro phosphorylation (3). To obtain adequate incorporation of radioactive phosphate into protein and to control the calcium concentration,

polyethylene glycol was eliminated from all steps of purification, and during the phosphorylation reaction itself vanadate was used to inhibit the depletion of ATP by the dynein ATPases (11), and the concentration of ATP was lowered to preserve a high specific radioactivity. Under these modified conditions the specific radioactivity of the proteins phosphorylated with γ-^{32}P-ATP was sufficient to analyze the phosphorylated proteins by one- and two-dimensional gel electrophoresis. However, none of these axonemes are motile.

Time course experiments have shown that the overall level of radioactivity incorporated into protein during the in vitro phosphorylation, as judged by one-dimensional SDS PAGE, increases from 0 to 2 min, is stable from 2 to 5 min, then decreases rapidly on further incubation. The in vitro phosphorylation reaction was therefore standardized for 5-min incubations; this gave a sample of axonemes labeled to a specific radioactivity of 3,000–14,000 cpm/μg.

The overall pattern of axonemal proteins phosphorylated in vitro, as assessed by two-dimensional gel analysis, was consistent and highly reproducible. A typical pattern is shown in Fig. 1. Autoradiograms such as this one were compared with the autoradiograms of two-dimensional electrophoretograms of axonemal proteins phosphorylated in vivo by a 10-min pulse with ^{32}P-phosphate (see reference 49). While the overall patterns were quite different, many of the proteins phosphorylated in vitro could be matched up with in vivo phosphoproteins on the basis of molecular weight and isoelectric mobility. A perfect one to one correlation between in vivo and in vitro phosphoproteins was not possible. The similarities between the in vivo and in vitro preparations of axonemal phosphoproteins were studied systematically. Several axonemal polypeptides, constituents of the radial spokes (16, 39), the central microtubule pair (1), or the B-subfiber projections (49), have been shown to be phosphorylated in vivo. The phosphoproteins could be readily identified in the autoradiograms of in vitro phosphorylated axonemes, by comparing the autoradiograms of wild-type, where they were present, with the mutant axonemes phosphorylated in vitro, where they were not. Axonemes were prepared from *pf18* mutants, which lack the central pair microtubules (1, 43, 44,

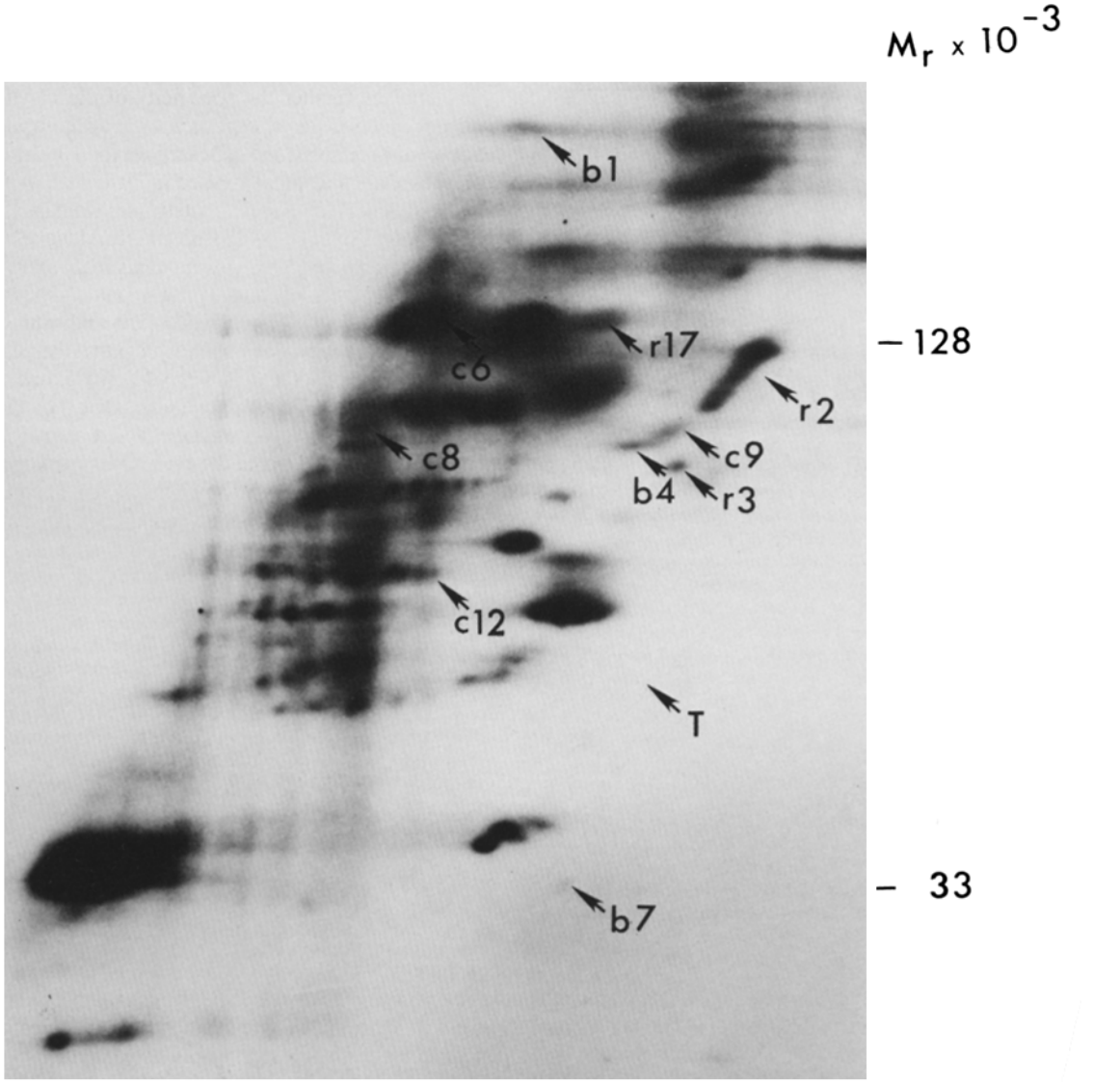

FIGURE 1 Autoradiogram of two-dimensional SDS PAGE of wild-type axonemes (137c) phosphorylated in vitro. The axonemal proteins were labeled to a specific radioactivity of 8,000 cpm/μg. The phosphoproteins identified by in vitro phosphorylation studies using the mutants *pf18*, *pf27*, and *mbo2* are indicated by arrows and the appropriate designations.

54), from *pf27*, which have defects in the phosphorylation of radial spoke proteins (16), and from *mbo2* mutants, which lack the B-subfiber projections (49). Each of these axonemal samples was phosphorylated in vitro, and the resultant phosphoproteins were analyzed by two-dimensional gel electrophoresis. The autoradiographic patterns were compared with wild-type axonemes phosphorylated in vitro and with the mutant axonemes phosphorylated in vivo. These experiments identified many of the polypeptides phosphorylated in vitro; these proteins are indicated with appropriate designations (reviewed in reference 28) in Fig. 1. Table I shows a comparison between the phosphoprotein deficiencies observed in vivo and in vitro using these mutants. Ten of the sixteen phosphoproteins were phosphorylated in vitro. The six proteins whose in vitro phosphorylation could not be detected were generally less prominent phosphoproteins in vivo.

The experiments with the mutants demonstrated that many of the same proteins were phosphorylated in vitro and in vivo.

TABLE I. *Phosphorylated Axonemal Components*

Structure	Polypeptide component	$M_r \times 10^{-3}$	Labeled with ^{32}P In vivo (10 min)	Labeled with ^{32}P In vitro (5 min)
Central pair microtubules	c5	142	+	−
	c5	128	+	+
	c8	97	+	+
	c9	97	+	+
	c12	66	+	+
	c16	45	+	−
Radial spokes	r2	118	+	+
	r3	86	+	+
	r5	69	+	−
	r13	98	+	−
	r17	124	+	+
B-Subfiber projections	b1	245	+	+
	b4	95	+	+*
	b5	88	+	−
	b6	55	+	−
	b7	33	+	+

* In $\geq 10^{-5}$ M Ca^{2+}.

To determine if the substrate specificity for the phosphorylation reactions was impaired in vitro, the phosphorylation of tubulin was studied. Tubulin constitutes 70% of the axonemal protein mass (26, 40) and is not phosphorylated in vivo. Thus, the presence of phosphorylated tubulin is a sensitive indicator of nonspecific phosphorylation. Using the in vitro system, only a very low level of tubulin phosphorylation was detected when the samples were analyzed on two-dimensional gel electrophoresis. In the autoradiogram shown in Fig. 1, the tubulin, indicated by the letter T, is a very faint spot. This was identified by its correspondence to the major Coomassie Blue–stained area of this electrophoretogram.

The in vitro phosphorylation system using the axonemes prepared by dibucaine deflagellation was compared to other in vitro systems. The specificity of these other systems could be assessed by observing the extent of tubulin phosphorylation. Phosphorylation systems using whole flagella after deflagellation by pH shock (9, 54), or the same flagella lysed with 0.1% Nonidet P-40 at the time of initiating the phosphorylation reaction, or the same flagella ruptured by homogenization, or axonemes prepared from these flagella, resulted in a much higher level of incorporation of phosphate into tubulin. Therefore, all other experiments were done using the dibucaine system.

To analyze further the specificity of the in vitro phosphorylation system, the phosphoamino acid composition of the axonemal proteins from cells labeled for 10 min in vivo with ^{32}P-phosphate and those labeled in vitro with γ-^{32}P-ATP were analyzed. Total axonemal protein was prepared, hydrolyzed, and analyzed by a two-dimensional thin layer electrophoresis system (17). The phosphoamino acids from axonemes labeled in vivo, shown in Fig. 2*A*, contain predominantly phosphoserine, with a smaller amount of phosphothreonine and a very small quantity of phosphotyrosine. The phosphoamino acid composition of the in vitro labeled axonemal proteins, shown in Fig. 2*B*, is similar. Quantitation of the phosphoamino acids was done by cutting out and counting each of the phosphoamino acids from in vivo and in vitro phosphorylated samples. The results are shown in Table II and indicate no significant differences in the ratios between the phosphoamino acids in the two samples.

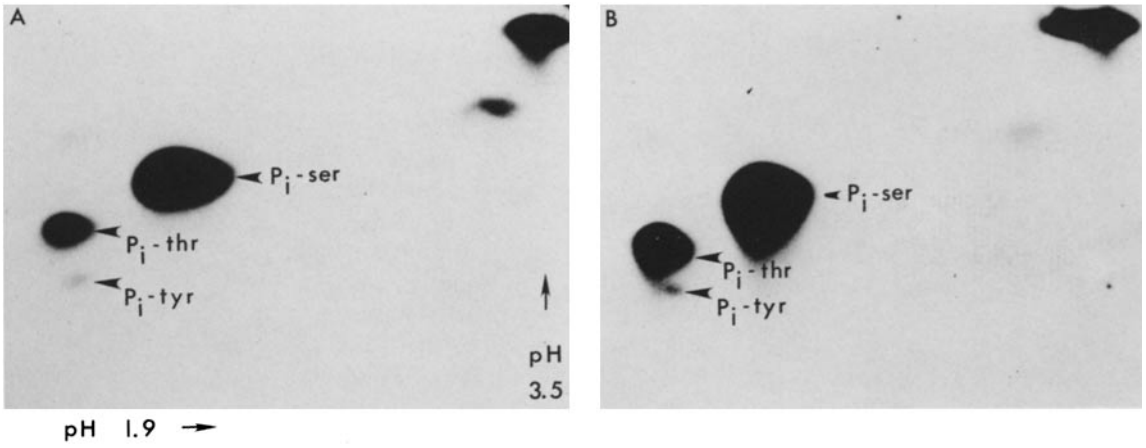

FIGURE 2 Phosphoamino acid analysis of axonemal proteins from wild-type (137c) cells labeled in vivo (*A*) and in vitro (*B*). The positions of phosphoserine, phosphothreonine, and phosphotyrosine determined by the ninhydrin-stained standards are indicated. The samples were first electrophoresed in the horizontal direction at 1 kV for 1½ h in pH 1.9 buffer, then in the vertical direction at 0.75 kV for 1 h in pH 3.5 buffer.

Effects of Ca^{+2} on Phosphorylation

The in vitro phosphorylation system allowed us to study the effects of Ca^{+2} on the axonemal kinases and phosphatases, as illustrated in Fig. 3. Equal aliquots of a wild-type axonemal suspension were labeled in vitro with ^{32}P-ATP in Ca^{+2}-EGTA buffered systems maintaining the free calcium ion concentration at 5×10^{-4} M (Fig. 3*A*) and at 5×10^{-8} M (Fig. 3*B*). The complete samples were then analyzed in parallel by two-dimensional SDS PAGE. The electrophoretograms were visualized by autoradiography for equal lengths of time. While most proteins are not affected by calcium concentration, the phosphorylation of two polypeptides are consistently affected by calcium concentration. As seen in these representative autoradiograms the phosphorylation of a 95,000-mol-wt protein, designated b4 (see below), is greatly stimulated by the presence of calcium ions, while the phosphorylation of a second 85,000-mol-wt phosphoprotein, indicated by the small black arrows, is greatly decreased in high concentrations of Ca^{+2}. The phosphorylation of other proteins is not influenced by Ca^{+2} concentration, as exemplified by the two proteins b1 and b7. To determine whether the difference in the phosphorylated protein was due to a calcium effect on the phosphorylation reaction or was an artifact caused by a difference in the entry of the protein into the gel system, the electrophoretograms of axonemes phosphorylated in vitro in low and in

TABLE II. *Comparison of Phosphoamino Acid Content of Axonemal Proteins Phosphorylated*

	In vivo	In vitro
Phosphoserine	93.6 ± 0.4	89.3 ± 1.4
Phosphothreonine	5.9 ± 0.2	8.5 ± 0.8
Phosphotyrosine	0.5 ± 0.1	1.2 ± 0.6

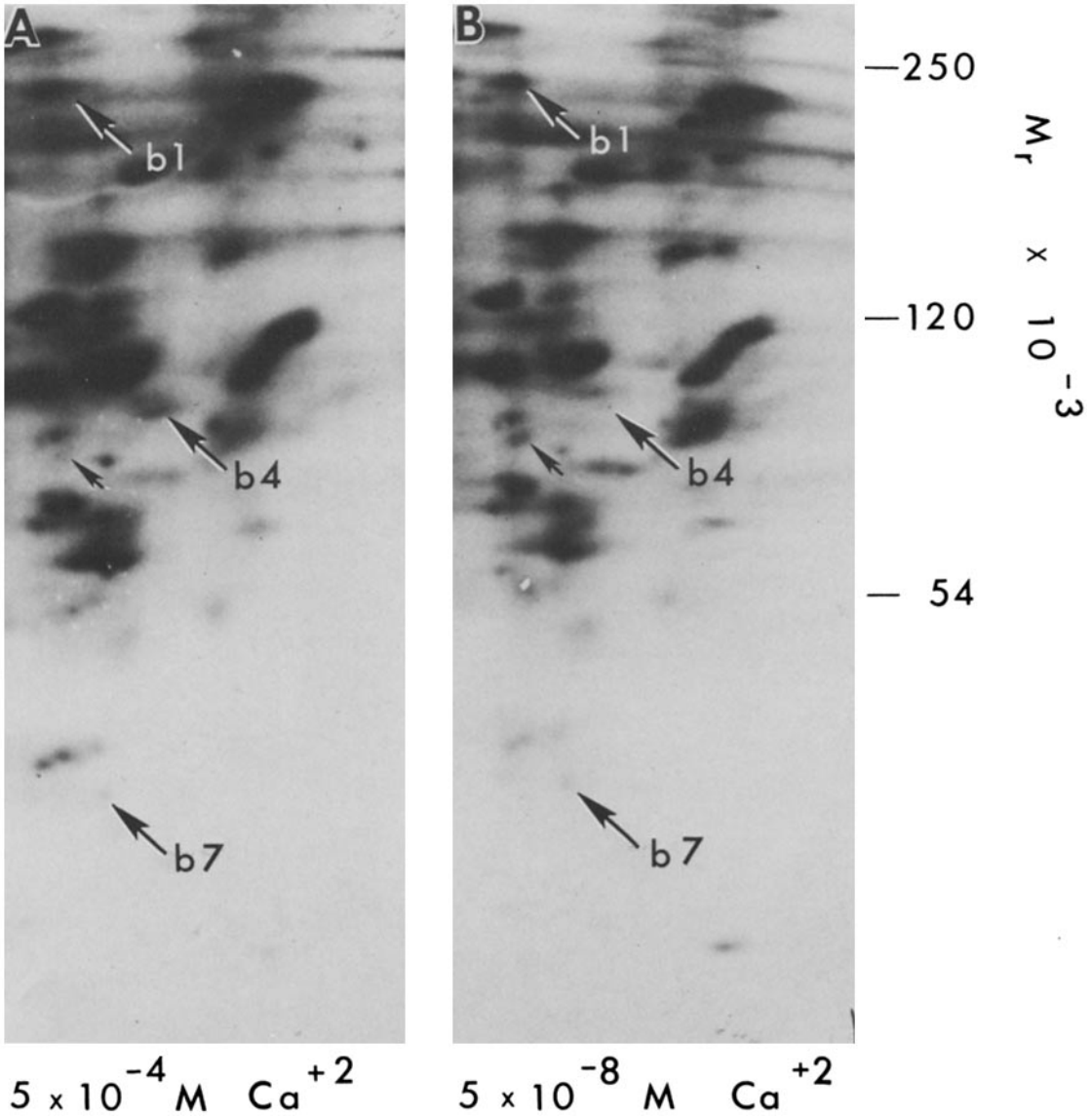

FIGURE 3 Portions of autoradiograms of two-dimensional SDS PAGE of wild-type axonemes (137c) phosphorylated in vitro in 5×10^{-4} (*A*) and 5×10^{-8} M Ca^{+2} (*B*). The axonemal proteins were labeled to a specific radioactivity of 8,000 cpm/μg. The large arrows indicate the 95,000-mol-wt polypeptide, *b4*, whose phosphorylation is stimulated by Ca^{+2}, and the small black arrows indicate the 85,000-mol-wt protein whose phosphorylation is inhibited by Ca^{+2}. These phosphoproteins are more prominent in *A* and in *B*, respectively. The phosphorylation of proteins b1 and b7 is unchanged by Ca^{+2}, and therefore the intensities of these proteins are equal in *A* and *B*.

high calcium were silver stained. While the relative mass of the 95-kD protein is the same in the low calcium (Fig. 4*A*) and in the high calcium (Fig. 4*B*) buffers, this protein was clearly more prominent in the ^{32}P-autoradiogram of the high calcium that in the low calcium sample (data are similar to Fig. 3). Therefore, the phosphorylation of this polypeptide is stimulated by calcium. The 85-kD protein whose phosphorylation is decreased in high calcium is not detectable by the silver stain.

To ascertain whether the calcium-dependent phosphorylation reactions could be a site for the calcium stimulation of the photophobic response, b4 phosphorylation was studied as a function of calcium concentration. The in vitro phosphorylation of b4 showed essentially the same concentration dependence as did the induction of backward movement in vitro. When Bessen and co-workers studied the percentage of reactivated axonemes that use a flagellar stroke as a function of calcium concentration, they found that the transition from the ciliary to the flagellar-type stroke occurred at calcium concentrations between 10^{-6} and 10^{-4} M (3). In three experiments, wild-type axonemes were phosphorylated in vitro at Ca^{+2} concentrations calculated at 5×10^{-8}, 5×10^{-6}, 5×10^{-5}, and 5×10^{-4} M. The stimulation of b4 phosphorylation is maximal at calcium concentrations greater than 5×10^{-6} M. This is shown in Fig. 5; b4, indicated by the large arrows, is maximally phosphorylated at 5×10^{-4} or 5×10^{-5} M Ca^{+2} (panels *A* and *B*). The intensity of this spot is quite low at 5×10^{-6} M, and is barely detectable at 5×10^{-8} M Ca^{+2}.

The phosphorylation of the unidentified protein of molecular weight 85,000 affected by calcium can also be studied as a function of Ca^{+2} concentration. This protein, indicated by the small black arrows in Fig. 5, is maximally phosphorylated at 5×10^{-8} M. The extent of the phosphorylation decreases sharply at 5×10^{-6} M, and the phosphorylation is not detectable at higher Ca^{+2} concentrations. In this figure the phosphorylation of a M_r 70,000 protein appears to be stimulated by the Ca^{+2} concentration. However, this protein showed variable phosphorylation that did not consistently depend on the calcium ion concentration.

The reactivation experiments of Bessen et al. (3) and of Hyams and Borisy (18, 19) demonstrated that the axonemal beat pattern can change within milliseconds of an alteration in calcium concentration. Since axonemes prepared for analysis of in vitro phosphorylation in the absence of polyethylene glycol and in the presence of vanadate cannot be reactivated, we could not determine the kinetics of the reversal response

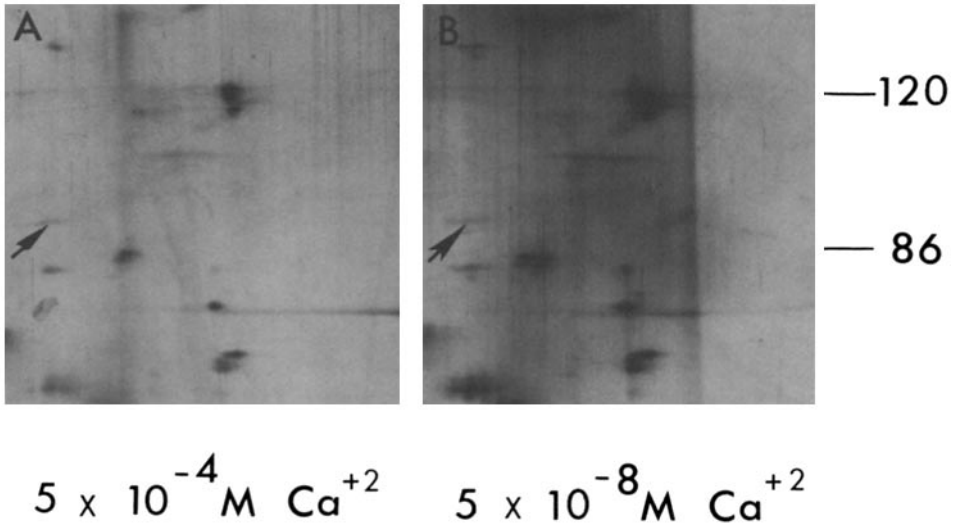

FIGURE 4 Portions of electrophoretograms of two-dimensional SDS PAGE of wild-type axonemes (137c) phosphorylated in vitro in 5×10^{-4} (*A*) and 5×10^{-8} (*B*) M Ca^{+2} and visualized by silver staining. The 95,000-mol-wt protein, b4, indicated by the arrows, is equally prominent in panels *A* and *B*.

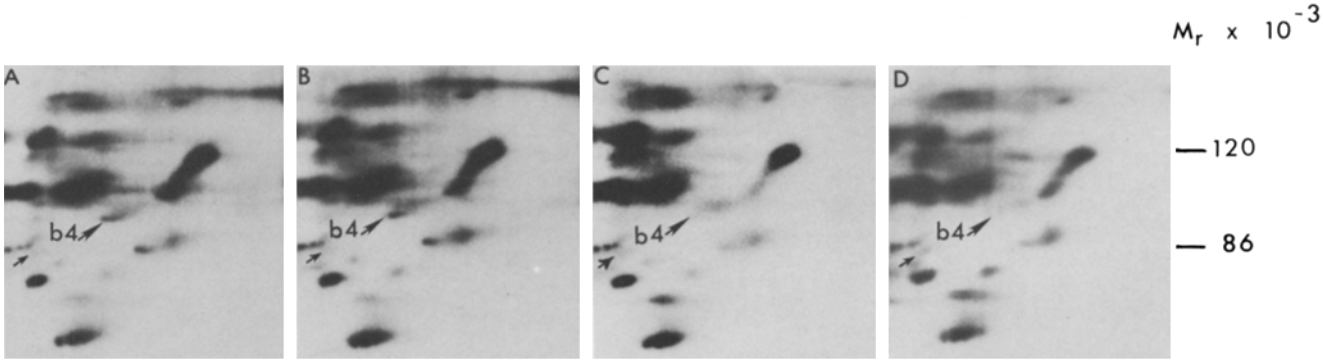

FIGURE 5 Portions of autoradiograms of two-dimensional SDS PAGE of wild-type axonemes phosphorylated in vitro in 5×10^{-4} m Ca^{+2} (*A*), 5×10^{-5} M Ca^{+2} (*B*), 5×10^{-6} M Ca^{+2} (*C*), and 5×10^{-8} M Ca^{+2} (*D*). The axonemal proteins were labeled to a specific radioactivity of 4,000 cpm/μg. The 95,000-mol-wt protein, indicated by the large arrow, is maximally phosphorylated in *A* and *B*, and the phosphorylation gradually decreases as Ca^{+2} concentration decreases. The phosphorylation of the 85,000-mol-wt protein indicated by the small arrows is inhibited in panel *A*, and the phosphorylation increases only in panels *C* and *D*.

in our system. We did attempt to study phosphorylation at short intervals but could detect calcium-stimulated phosphorylation of b4 only after 2 min.

Identification of the 95-kD Polypeptide

The identification of the 95,000-kD phosphoprotein as polypeptide b4 is illustrated in Fig. 6. While this protein had the same isoelectric mobility and molecular weight as b4, the *mbo* mutants were necessary for the definitive identification of this protein. Axonemes were prepared from wild-type and *mbo2* cells, and equal aliquots were phosphorylated in vitro in 5×10^{-4} M and 5×10^{-8} M Ca^{+2}, and analyzed by two-dimensional gel electrophoresis. The resultant autoradiograms were compared with each other and with the autoradiograms of wild-type and *mbo2* axonemes prepared from cells pulse-labeled with ^{32}P-phosphate and analyzed by two-dimensional gel electrophoresis. As shown above, the 95-kD protein is much more prominent in the sample of wild-type axonemes phosphorylated in 5×10^{-4} M Ca^{+2} (Fig. 6*A*) than in 5×10^{-8} M Ca^{+2} (Fig. 6*B*). The same protein cannot be detected in the *mbo2* axonemes phosphorylated at high calcium (Fig. 6*C*) or at low calcium (Fig. 6*D*). This phosphoprotein is prominent in wild-type axonemes phosphorylated in vivo (Fig. 6*E*) and is absent from the *mbo2* in vivo phosphorylated axonemes (Fig. 6*F*). These data establish that the 95-kD phosphoprotein is indeed b4.

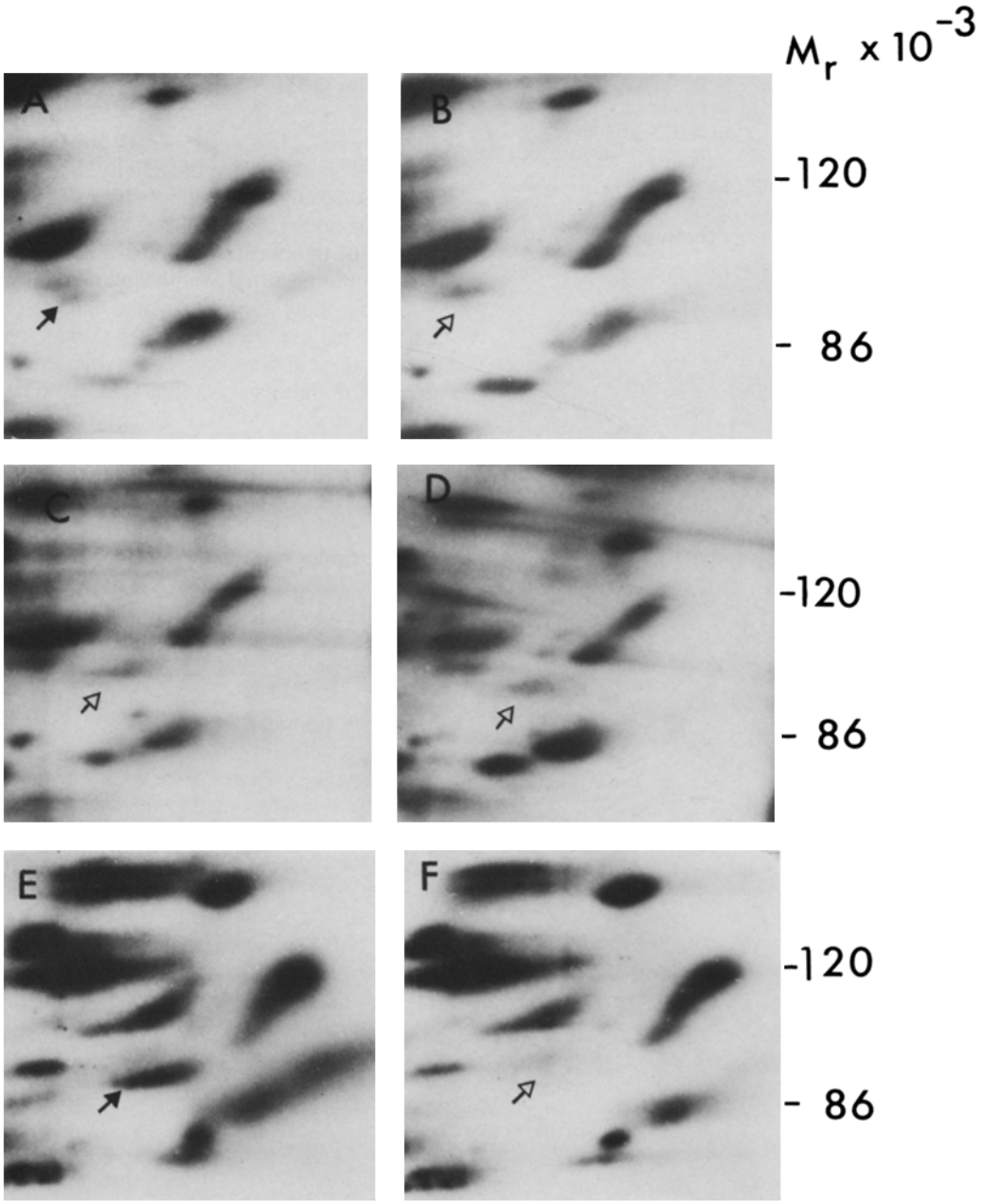

FIGURE 6 Portions of autoradiograms of two-dimensional SDS PAGE of wild-type (*A* and *B*) and *mbo2* axonemes (*C* and *D*) phosphorylated in vitro in 5×10^{-4} M (*A* and C) and 5×10^{-8} M Ca^{+2} (*B* and *D*). The arrows in *A* and *B* and the open arrowheads in C and *D* indicate the 95,000-mol-wt polypeptide b4. There is no protein whose phosphorylation is stimulated by Ca^{+2} in this mutant. Panels *E* and *F* show the corresponding portions of autoradiograms of two-dimensional SDS PAGE of wild-type (*E*) and *mbo2* (*F*) axonemal proteins labeled in vivo with ^{32}P-phosphate. The arrow (*E*) and open arrowhead (*F*) once again indicate polypeptide b4.

The *mbo* mutants are deficient in several other phosphoproteins, which can be identified in the electrophoretograms of the axonemes phosphorylated in vitro. The level of phosphorylation of these polypeptides, indicated as b1 and b7 in Fig. 3, does not depend on the calcium concentration. Thus, the Ca^{+2} stimulation of phosphorylation is specific to the b4 polypeptide.

Effects of Kinase Activators and Inhibitors on Phosphorylation

In an attempt to identify a kinase that might be responsible for the b4 phosphorylation, the effects of molecules able to influence cyclic nucleotide–dependent kinases, the calcium-calmodulin–dependent kinases, and the C kinase on the in vitro phosphorylation of wild-type axonemes were studied. All reagents were assayed both at 5×10^{-4} M and 5×10^{-8} M Ca^{+2}. The samples were analyzed by two-dimensional SDS PAGE, and the autoradiograms from reactions in high and low calcium were compared with each other and with normal controls similar to Fig. 3. None of the agents tested detectably altered the phosphorylation of b4 or of other identified proteins. The reagents tested and the concentrations used are listed in Table III.

Phosphoamino Acid Analysis of b4

The residue phosphorylated by a kinase is generally specific and characteristic. Therefore, to characterize further the calcium-dependent phosphorylation of polypeptide b4, the residue(s) on which b4 is phosphorylated was determined. Because of the low levels of incorporation of radioactivity into protein in the in vitro system, the phosphoamino acid composition of individual polypeptides could only be analyzed using axonemes labeled in vivo. Phosphoserine was the only phosphorylated residue detected after hydrolysis of the radioactively labeled b4 polypeptide isolated by two-dimensional gel electrophoresis (data not shown).

TABLE III. *List of Reagents Tested*

Reagent	Concentration tested
A. Cyclic nucleotide kinase activators and inhibitors	
Caffeine	1 mM
cAMP	50 μM
Caffeine and	1 mM
Dibutyryl cAMP	20 μM
Caffeine and	1 mM
Dibutyryl cGMP	20 μM
Protein inhibitor of cAMP-dependent kinase	1 μg/ml
B. Calmodulin and kinase C inhibitors	
2 Norchlorpromazine	5 μM; 50 μM
Trifluoperazine	50 μM
Calmidazolium (R241752)	10 μM
W-7	50 μM
(Control) W-5	50 μM
C. Activators of calmodulin-dependent activities	
Calmodulin	0.5 μg/ml
D. Activators of kinase C	
L-Phosphatidyl serine	0.5 μg/ml
L-Phosphatidyl serine and	10 μg/ml
Phorbol 12 myristate 13 acetate 4-O-methyl ether	10 μg/ml
E. Myosin light chain kinase substrate and inhibitors	
Peptide substrate: KM14SM	100 μM
Peptide inhibitor: KM14A3	100 μM

All reagents tested did not affect Ca^{2+}-dependent phosphorylation of b4.

Axonemal Protein Thiophosphorylation

In an attempt to distinguish the effects of calcium on phosphorylation from effects on protein dephosphorylation, wild-type axonemes were phosphorylated in vitro in high and low calcium, using ATPγS instead of ^{32}P-ATP. ATPγS is a poor substrate for ATPases, and in general it can substitute for ATP in kinase reactions much more readily than the resultant thiophosphorylated protein can serve as a substrate for protein phosphatases (5, 13). Consistent with previous studies using ATPγS (5, 13), the time course of thiophosphorylation of axonemal proteins was found to be different from the time course of axonemal protein phosphorylation. The overall level of ^{35}S-radioactivity incorporated into protein, as assessed by measuring trichloracetic acid–precipitable counts, increases gradually from 0 to 30 min. After the 30-min peak of thiophosphorylation, a slow decrease in incorporated counts occurred over the following 30 min. Therefore, the thiophosphorylation of isolated axonemes was standardized at 30 min, rather than the 5-min incubation used for axonemal protein phosphorylation. No vanadate was added during the thiophosphorylation reaction. The concentration of ATPγS used (5 μM) was 20-fold lower than the standard concentration of ATP used for phosphorylation; the specific radioactivity of the ATPγS was 11,000 cpm/pmol, which is comparable to that of the ^{32}P-ATP used. Under these conditions, the radioactivity incorporated into protein at 30 min was approximately 10 times lower with the thioATP. Fig. 7 shows an autoradiogram of wild-type axonemes thiophosphorylated in vitro in 5×10^{-4} M (*A*) and 5×10^{-8} M (*B*) Ca^{+2}, and analyzed by two-dimensional SDS PAGE. The overall pattern of thiophosphorylated proteins in the absence of vanadate is quite similar to that seen with γ-^{32}P-ATP in the presence of vanadate (compare Fig. 7*A* to Fig. 3). Several identified phosphoproteins, including r2, r3, r17, c6, c8, c12, and b7, could apparently be seen in the autoradiograms of thiophosphorylated axonemes. However, some polypeptides, notably b1, b4, and c9, were not detectable in the autoradiograms. Therefore, we could not examine the effect of calcium concentration on the thiophosphorylation of b4. The M_r 85,000 phosphoprotein can be seen in the axonemal sample thiophosphorylated in 5×10^{-8} M Ca^{+2} and is indicated by the solid arrow in Fig. 7*B*. This component cannot be detected in the sample thiophosphorylated in 5×10^{-4} M Ca^{+2}, and the corresponding deficiency is indicated by an open arrowhead in Fig. 7*A*.

DISCUSSION

A system in which to study the phosphorylation of axonemal proteins in vitro has been established and characterized. The in vitro phosphorylation reactions observed showed good fidelity to what has been observed in vivo. This system was used to identify a phosphorylation reaction that may be involved in the photophobic response of *Chlamydomonas reinhardtii*. This photophobic response is a calcium-mediated reversal reaction in which the organism transiently alters its

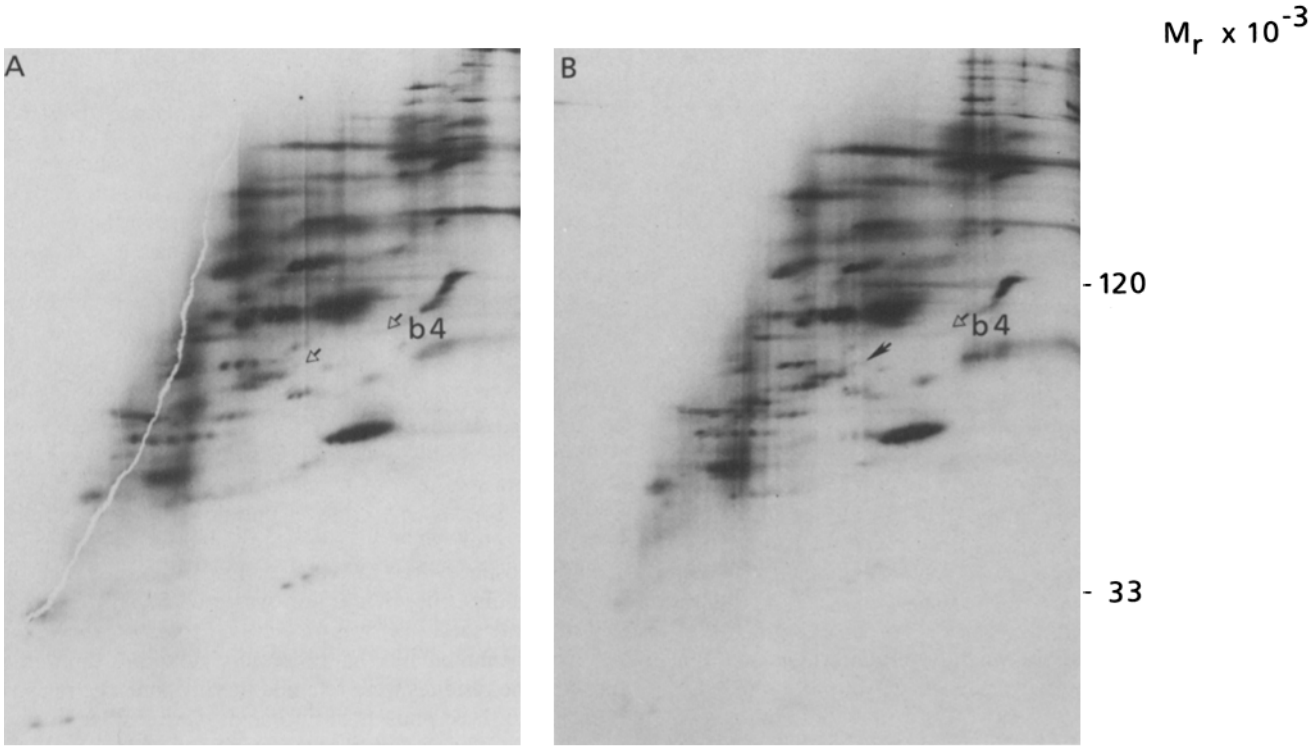

FIGURE 7 Autoradiograms of two-dimensional SDS PAGE of wild-type axonemes thiophosphorylated for 30 min in vitro with ATP-^{35}S in 5×10^{-4} M (*A*) and 5×10^{-8} M (*B*) Ca^{+2}. b4 cannot be seen in either panel. The position normally occupied by b4 is indicated with open arrowheads in both panels. The 85,000-mol-wt phosphoprotein is detectable in panel *B* (arrow) but is almost undetectable in panel *A* (open arrowhead).

axonemal beat pattern from an asymmetrical to a symmetrical beat pattern (45, 47). While the molecular basis for the reversal is not understood, the *mbo* mutants have identified six polypeptides that may have a role in the change in waveform involved (49). The finding that one of these six polypeptides, b4, is a phosphoprotein whose in vitro phosphorylation is stimulated by calcium suggests the possibility that this phosphorylation is a link between the increase in intracellular calcium concentration and the reversal response.

Effect of Calcium on Phosphorylation

Calcium was consistently found to affect the in vitro phosphorylation of two axonemal polypeptides. This divalent cation reproducibly stimulates the phosphorylation of the identified protein b4, while it represses the phosphorylation of a previously unidentified M_r 85,000 protein. The incorporation of phosphate into other proteins was not affected by calcium ions. The observation that calcium stimulates the b4 phosphorylation with essentially the same concentration dependence as it stimulates the switch from an asymmetrical to a symmetrical stroke (3) supports the possibility that this phosphorylation is a part of the mechanism for the calcium-mediated change in waveform. The *mbo* mutants have a deficiency in protein b4 and are unable to alter the axonemal waveform in response to calcium (49). If calcium-stimulated phosphorylation acts to repress the activity of b4 during the photophobic response, then the observed deficiency of this polypeptide in the *mbo* mutants might, in part, account for the *mbo* motility defect.

The effect of calcium on b4 phosphorylation could be due to calcium-stimulation of a kinase, or calcium-repression of a phosphatase. While many calcium-dependent kinases have been characterized (reviewed in references 7, 24, 35, and 36), no calcium-repressed phosphatases have yet been described. Therefore, at present, it seems more likely that a kinase is the calcium control site for b4 phosphorylation. There is no detectable phosphorylation of b4 when ATP is replaced by ATPγS, and so we cannot yet experimentally distinguish between a b4 kinase reponsive to calcium and a b4 phosphatase controlled by the divalent cation.

Since calmodulin has been shown to be present in the *Chlamydomonas* axoneme (12, 46, 51), the possibility that the b4 phosphorylation involves a calcium-calmodulin–dependent enzyme was explored. Many calcium-calmodulin–dependent kinases have been characterized (14, 22, 35, 42, 50, 52, 53) and shown to be inhibited by low to moderate concentrations of calmodulin inhibitory drugs (2, 37, 50, 52). The calcium-stimulated phosphorylation of b4 was not detectably repressed by trifluoperazine or other calmodulin-binding drugs, nor did heterologous calmodulin result in increased stimulation of phosphorylation (Table III). This evidence argues against the idea that the b4 kinase is calmodulin dependent. The possibility that calmodulin is the calcium-binding protein responsible for mediating the stimulation of b4 phosphorylation cannot, however, be excluded. If the inhibitors only have a partial effect on the level of b4 phosphorylation, the present, nonquantitative assay would not detect the difference. Attempts to quantitate the amount of b4 phosphorylation have not been successful due to the low amount of radioactive b4 in individual gels and the close proximity of other ^{32}P-labeled components (notably c9). Furthermore, if the calmodulin is tightly bound to the kinase, as is true for phosphorylase kinase (50, 52), the calmodulin

inhibitor drugs might not affect the enzymatic activity. Finally, some protein or other axonemal component may bind these drugs and so reduce the effective drug concentration.

The possibility that b4 phosphorylation involves the C kinase, the other characterized type of calcium-dependent kinase (reviewed in reference 36), was also considered. This calcium-phospholipid–dependent enzyme is unlikely to be the b4 kinase since C kinase stimulators, such as phosphatidyl serine and a phorbol ester (6), do not enhance b4 phosphorylation. Furthermore, many drugs that inhibit the C kinase, such as the phenothiazines (56), did not repress b4 phosphorylation. The fact that the C kinase is usually associated with cell membranes (23), while b4 appears to be located within the lumina of the outer doublets (49) far from any membrane, further decreases the likelihood that the C kinase phosphorylates b4.

It is possible that the b4 phosphorylation involves a novel sort of calcium-dependent kinase—one that does not require either calmodulin or a phospholipid. To prove this conclusively, it would be necessary to purify and characterize the b4 kinase.

Calcium has no effect on the in vitro phosphorylation of the polypeptides b1 and b7, which implies that there is not a calcium-stimulated cascade of phosphorylation of b-polypeptides. Instead, this suggests that b4 may have a unique early role in effecting the reversal reaction. Unfortunately, it is not possible to show that the calcium stimulation of b4 phosphorylation also occurs in vivo during the photophobic response. Under our usual labeling condition, cells in low calcium (10^{-7} M) take up only 20% of the ^{32}P-phosphate, while cells in the normal medium, containing 1 mM $CaCl_2$, take up >99% of the radioactivity. Furthermore, in low calcium, cells cannot be effectively deflagellated either by pH shock or by treatment with dibucaine. Therefore, for the moment, the calcium effect can only be observed in vitro.

At present, the significance of the M_r 85,000 protein whose phosphorylation is depressed by calcium is unclear. The effect of calcium on the ^{32}P-labeling of this protein could be mediated by a specific kinase, phosphatase, or protease. The fact that the calcium dependence is observed using ATPγS argues against the involvement of a phosphatase. The data could be explained by a calcium-dependent protease specific for the 85-kD protein, or by a specific kinase that is directly or indirectly inhibited by calcium. While calcium-dependent proteases with substrate specificity have been described (30, 34), it seems unlikely that such an enzyme would have a sufficiently high degree of substrate specificity that it would cleave only one axonemal protein, present at very low concentration relative to total protein. The results might also be due to a kinase inhibited by calcium. While such a kinase could be important for the photophobic response, this potential calcium control point could just as well be involved in maintaining internal calcium concentration, in phototaxis (20), or in some other calcium-mediated process.

In Vitro Phosphorylation System

To assess the significance of the in vitro effect of Ca^{+2} on b4 phosphorylation, the phosphorylation system must be evaluated. While the axonemes used for the in vitro phosphorylation are prepared by a protocol similar to that used in preparing motile axonemes able to execute both ciliary and flagellar-type waveforms (3), the effects of the omission of polyethylene glycol from the preparation, and of the addition of vanadate and a calcium–EGTA buffer to the final axonemal preparation are not known. Certainly, the vanadate-induced inhibition of the normal dynein ATP-hydrolyzing cycle of attachment to the adjacent doublet, microtubular sliding, and detachment, may alter many of the phosphorylation/dephosphorylation reactions that occur in actively beating axonemes. However, the similarity between the proteins labeled with ATPγS in the absence of vanadate and those labeled with ^{32}P-ATP in the presence of the inhibitor implies that any effect of the vanadate on the phosphorylation reactions observed is minor.

While the overall patterns of proteins phosphorylated in vivo and in vitro do differ, many polypeptides phosphorylated in vitro were identified as in vivo phosphoprotein components of central pair, radial spokes, and the B-subfiber projections. The substrate specificity of phosphorylation in vitro was further evaluated by observing the phosphorylation of tubulin. While this is the major protein component of the axoneme, it is not phosphorylated in vivo. The fact that only a very low level of tubulin phosphorylation was seen in vitro suggests that substrate specificity was preserved. Moreover, the activities of axonemal kinases that phosphorylate serine, threonine, and tyrosine residues were retained in vitro, and the proportions of the three phosphoamino acids were similar in vivo and in vitro. This suggests that the specificity of the phosphate attachment site is maintained in vitro.

It is interesting that phosphotyrosine is present in the axoneme, and that a kinase that phosphorylates tyrosine residues is also present in the axonemal preparations. Phosphorylation at tyrosine residues is generally correlated with oncogenic transformation (10, 17) or with response to growth factor stimulation (8). Several studies have suggested that some cytoskeletal proteins are phosphorylated at tyrosine residues (4, 48), but the function of such phosphorylation is unknown. The axoneme might represent a good system for studying microtubular-associated protein tyrosine phosphorylation. The axonemal protein(s) that are phosphorylated at tyrosine residues have not yet been identified. To date, phosphoamino acid analysis has been done on proteins r2, r3, b4, and b7; all are phosphorylated at serine residues, and r3 also contains a phosphothreonine (unpublished observations). In the future, identification of the phosphotyrosine-containing polypeptide might clarify the functional significance of the axonemal tyrosine kinase.

While the in vitro phosphorylation system has been used here primarily to study calcium control of axonemal phosphorylation, this system may prove useful for studying cytoskeletal tyrosine kinases and other aspects of axonemal assembly and motility. The characterization of the in vitro system indicates that many of the kinases, and at least some of the phophatases, that phosphorylate axonemal proteins are present in the flagella rather than in the cell bodies. The detergent-extracted flagella used for the in vitro phosphorylation reaction consist primarily of axonemes, however residual membrane and matrix components are still present in these preparations. Therefore, in the intact organism, the axonemal kinases could be primarily associated with the axoneme itself or may be located in the surrounding membrane and matrix. Kinases remained with axonemes prepared either by the modified dibucaine protocol or by pH shock, and axonemes extracted with high salt solutions (41) still retain many kinase activities, including the b4 kinase (unpublished observations).

These data suggest that some flagellar kinases, including the enzyme responsible for phosphorylating b4, may actually be a part of the axoneme structure.

The calcium-dependent phosphorylation of polypeptide b4 observed in vitro probably reflects an in vivo phosphorylation reaction, and may be one of the early axonemal events in the transition from a ciliary type stroke to a flagellar type stroke that occurs in the photophobic response. While the sequence of events involved in the reversal response in still unknown, the in vitro phosphorylation system has provided a new approach for studying this response. In combination with other approaches, especially genetic manipulation, it may in the future provide further information on the axonemal aspects of the photophobic response.

Received for publication 28 March 1985, and in revised form 4 July 1985.

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