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 Mr protein was repressed by calcium concentrations >10^{-6} M, while the phosphorylation of the previously identified 95,000 Mr 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.

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-
zations include small, triangular structures that are within the lumina of the B-subfibers of these doublets (15). The intraluminal structures were described by Hoops and Witman as bead-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. (Houston, TX), and the drug 2-norphchlorpromazine was a gift from Milton Cormier (La Jolla, CA), while the phosphoamino acid standards of calcium on protein phosphorylation. Calcium specifically

Phosphorylation in Isolated Chlamydomonas Axonemes

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-32P-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 32P-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](image-url)

**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.
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                  | Poly-peptide component | Labeled with 32P | In vivo (10 min) | 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 >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 32P-phosphate and those labeled in vitro with γ-32P-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. 2A, 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. 2B, 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.
Effects of \( \text{Ca}^{2+} \) on Phosphorylation

The in vitro phosphorylation system allowed us to study the effects of \( \text{Ca}^{2+} \) on the axonemal kinases and phosphatases, as illustrated in Fig. 3. Equal aliquots of a wild-type axonemal suspension were labeled in vitro with \( ^{32} \text{P}-\text{ATP} \) in \( \text{Ca}^{2+} \)-EGTA buffered systems maintaining the free calcium ion concentration at \( 5 \times 10^{-4} \) M (Fig. 3A) and at \( 5 \times 10^{-8} \) M (Fig. 3B). 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 is 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 \( \text{Ca}^{2+} \). The phosphorylation of other proteins is not influenced by \( \text{Ca}^{2+} \) 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

| 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 \( 5 \times 10^{-4} \) M \( \text{Ca}^{2+} \) (A) and \( 5 \times 10^{-8} \) M \( \text{Ca}^{2+} \) (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 \( \text{Ca}^{2+} \), and the small black arrows indicate the 85,000-mol-wt protein whose phosphorylation is inhibited by \( \text{Ca}^{2+} \). These phosphoproteins are more prominent in A and in B, respectively. The phosphorylation of proteins b1 and b7 is unchanged by \( \text{Ca}^{2+} \), 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. 4A) and in the high calcium (Fig. 4B) buffers, this protein was clearly more prominent in the 125I-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 $5 \times 10^{-8}$, $5 \times 10^{-6}$, $5 \times 10^{-5}$, and $5 \times 10^{-4}$ M. The stimulation of b4 phosphorylation is maximal at calcium concentrations greater than $5 \times 10^{-6}$ M. This is shown in Fig. 5; b4, indicated by the large arrows, is maximally phosphorylated at $5 \times 10^{-6}$ or $5 \times 10^{-5}$ M Ca$^{2+}$ (panels A and B). The intensity of this spot is quite low at $5 \times 10^{-8}$ M, and is barely detectable at $5 \times 10^{-6}$ 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 $5 \times 10^{-6}$ M. The extent of the phosphorylation decreases sharply at $5 \times 10^{-8}$ M, and the phosphorylation is not detectable at higher Ca$^{2+}$ concentrations. In this figure the phosphorylation of a M, 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 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.

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**Figure 4** Portions of electrophoretograms of two-dimensional SDS PAGE of wild-type axonemes (137c) phosphorylated in vitro in $5 \times 10^{-6}$ M Ca$^{2+}$ (A) or $5 \times 10^{-8}$ M Ca$^{2+}$ (B) 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 $5 \times 10^{-6}$ m Ca$^{2+}$ (A), $5 \times 10^{-4}$ M Ca$^{2+}$ (B), $5 \times 10^{-6}$ M Ca$^{2+}$ (C), and $5 \times 10^{-9}$ 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 $5 \times 10^{-4}$ M and $5 \times 10^{-8}$ 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 $5 \times 10^{-4}$ M Ca$^{2+}$ (Fig. 6A) than in $5 \times 10^{-8}$ M Ca$^{2+}$ (Fig. 6B). The same protein cannot be detected in the mbo2 axonemes phosphorylated at high calcium (Fig. 6C) or at low calcium (Fig. 6D). This phosphoprotein is prominent in wild-type axonemes phosphorylated in vivo (Fig. 6E) and is absent from the mbo2 in vivo phosphorylated axonemes (Fig. 6F). These data establish that the 95-kD phosphoprotein is indeed b4.

![Figure 6](image-url)

**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 $5 \times 10^{-4}$ M (A and C) and $5 \times 10^{-8}$ 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. All reagents tested did not affect Ca\(^{2+}\)-dependent phosphorylation of b4.

**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).

**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 ATP\(_{γ}\)S instead of \(^{32}\)P-ATP. ATP\(_{γ}\)S is a poor substrate for ATP\(_{γ}\)S, 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 ATP\(_{γ}\)S (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, increased 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 ATP\(_{γ}\)S used (5 μM) was 20-fold lower than the standard concentration of ATP used for phosphorylation; the specific radioactivity of the ATP\(_{γ}\)S 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. 7A 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, 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. 7B. 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. 7A.

**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...
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, 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 responsive 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 32P-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 ³²P-phosphate, while cells in the normal medium, containing 1 mM CaCl₂, 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 M₈₅,000 protein whose phosphorylation is depressed by calcium is unclear. The effect of calcium on the ³²P-labeling of this protein could be mediated by a specific kinase, phosphatase, or protease. The fact that the calcium dependence is observed using ATP₇S 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 Ca²⁺ 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 ATP₇S in the absence of vanadate and those labeled with ³²P-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 phosphatases, 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 part responsible for phosphorylating b4, may actually be a part of the axonemal 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.

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