Characterization of a Rapid, Blue Light-Mediated Change in Detectable Phosphorylation of a Plasma Membrane Protein from Etiolated Pea (Pisum sativum L.) Seedlings

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ABSTRACT

When crude microsomal membranes from apical stem segments of etiolated Pisum sativum L. cv Alaska are mixed in vitro with γ-[32P]ATP, a phosphorylated band of apparent molecular mass 120 kilodaltons can be detected on autoradiographs of sodium dodecyl sulfate electrophoresis gels. If the stem sections are exposed to blue light immediately prior to membrane isolation, this band is not evident. The response is observed most strongly in membranes from the growing region of the stem, but no 120 kilodalton radiolabeled band is detected in membranes from the developing buds. Fluence-response curves for the reaction show that the system responds to blue light above about 0.3 micro-mole per square meter, and the visible phosphorylation completely disappears above 200 micromoles per square meter. Reciprocity is valid for the system, because varying illumination time or fluence rate give similar results. If the stem segments are left in the dark following a saturating blue irradiation, the radiolabeled band begins to return after about 10 minutes and is as intense as that from the dark controls within 45 to 60 minutes. A protein that comigrates with the phosphorylated protein on polyacrylamide gels is also undetectable after saturating blue light irradiations. The fluence range in which the protein band disappears is the same as that for the disappearance of the phosphorylation band. Its dark recovery kinetics and tissue distribution also parallel those for the phosphorylation. In vitro irradiation of the isolated membranes also results in a phosphorylation change at that molecular mass, but in the opposite direction. Comparisons of the kinetics, tissue distribution, and dark recovery of the phosphorylation response with those published for blue light-mediated phototropism or rapid growth inhibition indicate that the phosphorylation could be linked to one or both of these reactions. However, the fluence-response relationships for the change in detectable phosphorylation match quite closely those reported for phototropism but not those for growth inhibition. Blue light has also been found to regulate the capacity for in vitro phosphorylation of a second protein. It has an apparent molecular mass of 84 kilodaltons and is localized primarily in basal stem sections.

These include phototropism, growth inhibition, stomatal opening, and a number of biochemical and enzymatic activity changes (see reviews in refs. 15, 20–22). In some of these reactions (e.g. nitrate reductase activity), blue light is thought to excite an integral chromophore in a reaction that directly alters the activity of the specific enzyme (17). In other cases, there is apparently a more complex sensory transduction chain acting between the initial absorption of quanta and its observable consequences in the organism. A few of these complex effects of blue light on photomorphogenesis have been described in detail (1, 5, 16). However, very little is understood about the biochemical events intervening between irradiation and the physiological responses. Part of the reason for this difficulty is that no one has been able to isolate a moiety that can be convincingly labeled a blue-light photoreceptor in plants or fungi; in fact there is considerable evidence to indicate that more than one type of blue-light photoreceptor molecule is present (6). Furthermore, many of the physiological responses to blue light—phototropic curvature and inhibition of growth, for example—are at least several minutes removed in time from the initial light reception event. Hence, it remains difficult to address questions about events early in the transduction chain and to differentiate between primary transduction events and their subsequent, indirect effects.

Since direct attempts at isolating a blue light receptor have been unfruitful so far, a logical alternative is to identify biochemical or biophysical modifications occurring as early as detectable following irradiation with blue light. Isolation and characterization of the constituents involved in such light-dependent events might be valuable both for identifying the responsible chromophore moiety and for elucidating early steps in a transduction chain to the associated physiological response.

In a previous paper (11), we described a system in which a brief in vivo blue light treatment alters the ability of a plasmalemma-associated protein to be phosphorylated in vitro. Since phosphorylation and dephosphorylation of proteins is a common means of regulating cellular protein activity (7, 8, 19), we performed a series of experiments to characterize further various aspects of the blue light-sensitive phosphorylation. In the present paper, we examine tissue distribution, kinetics, fluence-response, and other photobiological properties of the phosphorylation response and compare the results with some of the published findings for several physiological aspects.
reactions to blue light. A preliminary report of some of our findings appears elsewhere (23).

MATERIALS AND METHODS

Chemicals

Acrylamide, methylene-bis-acrylamide, and SDS were obtained from Serva (Westbury, NY). Tris and glycine (ultrapure) were from Bethesda Research Laboratories (Gaithersburg, MD), Mes was from Research Organics Inc. (Cleveland, OH), and sucrose (protease free) was from Boehringer (Indianapolis, IN). All other chemicals were standard enzyme grade obtained through Sigma.

Plant Material

Alaskan pea (Pisum sativum L., cv Alaska) seedlings were grown in total darkness for 7 d as described (11). Except as noted for the tissue distribution studies (see “Results”), standard stem segments of 8 to 10 mm were taken, starting 1 to 2 mm basal to the apical hook. Each sample normally consisted of 50 sections, and harvest of the standard eight samples (the maximum number for a given experiment) usually required less than 30 min. Since Gallagher et al. (11) showed that red light did not affect the phosphorylation phenomenon appreciably, segments were harvested under dim red light (approximately 0.5 μmol/m2s) at 24°C.

Light Sources

Red safelights were constructed from two Sylvania GTE red fluorescent bulbs (F20T12/R, 20W) enclosed in red plastic (Shinkolite 102, Argo Plastic Co., Los Angeles, CA.). The light source was covered with thin paper and positioned to give a fluence rate of 0.2 μmol/m2s at the bench surface. Broad spectral band blue light was obtained from a Kodak 760H projector with a 300W/120V ELH bulb (Eastman Kodak, Rochester, NY) and a 4 mm Corning CS 5-58 glass filter (Corning Glass Works, Corning, NY). The beam was passed through 15 mm of a 10% copper sulfate solution to minimize sample heating. Emission spectra, obtained with a Li-Cor Li-1800 Spectroradiometer (Lambda Instruments Corp., Lincoln, NE), indicated a single band between 400 and 480 nm with a maximum at 445 nm for this light source, with no other detectable emissions between 330 nm and 1100 nm. The light source provided a fluence rate at the samples of 8 μmol/m2s, as measured with a Li-Cor Li 185-A quantum photometer (Lambda Instruments) equipped with a calibrated Li-190S unidirectional quantum sensor. Intensity was adjusted as specified below with glass neutral-density filters (Balzers, Marlborough, MA). Irradiation times down to 0.1 s were controlled by a custom-built shutter system. For supersaturating light treatments employed in the dark recovery experiments, a custom-built theatrical light source with heat and blue filters as described (18) was used. This source produced blue light of spectral quality similar to that described above, but with a fluence rate at the samples of 300 μmol/m2s.

Irradiations

In vivo irradiations were performed with 50 plant segments floated horizontally on 5 mL distilled H2O in glass culture dishes. Care was taken to assure that sections did not shade one another. These were then irradiated from above. Following irradiation, the sections were either ground immediately in homogenization buffer (11) at 0 to 4°C or put into a dark box at 24°C and ground at 0 to 4°C after the appropriate dark incubation period indicated in Results. Dark control sections were treated in the same manner, except that they received no irradiation. In most cases a dark control was included both as the first and the last sample in order to judge any effect of time on in vitro phosphorylation. Repetitions of a given experiment were performed with the various treatments in different sequences to minimize the effect of any post-harvest, time-dependent variable. In vitro irradiations were performed on ice with 200 μg total protein in the phosphorylation buffer and distilled water mixture described for the phosphorylation reaction (11). The phosphorylation procedure was initiated immediately following each irradiation. Dark controls were left on ice for a comparable period of time prior to phosphorylation.

Membrane Isolation and Protein Separation and Analysis

Crude microsomal membrane fractions were prepared as in previous work (11), but the dim red safelights mentioned above were substituted for dim green during all isolation procedures. Extraction conditions, protein determination, in vitro phosphorylations, gel electrophoresis, autoradiography, and quantitation were all performed as described (11), except that instead of electrophoretic gels were immersed in a fixative solution (50% [v/v] EtOH, 10% [w/v] TCA) for 15 to 30 min, stained for 1 to 2 h (0.5% [w/v] Coomassie brilliant blue [R-250], 40% [v/v] MeOH, 7% [v/v] acetic acid), destained for 4 to 5 h (5% [v/v] EtOH, 7.5% [v/v] acetic acid), dried in a Bio-Rad 483 gel drier, and autoradiographed directly.

RESULTS

Tissue Distribution

Four regions of the pea epicotyls were chosen for tissue distribution studies: buds (including approximately half the apical hook), upper internodes (the topmost internode) 8 to 10 mm long as described above, upper nodes just below the topmost internode (containing 1–2 mm of internode tissue on either side), and lower internode segments 8 to 10 mm long harvested just below the upper node. In each experiment, two sets of each tissue type were harvested; one set was left in total darkness while the other was subjected to a 10 s pulse of blue light (80 μmol/m2s). The water was removed, and the tissue was immediately ground in ice-cold buffer. The membranes were isolated and phosphorylated, the proteins separated by SDS-PAGE with equivalent total protein loads, and the dried gels autoradiographed.

As can be seen in Figure 1A, there is negligible phosphorylation of a 120 kD protein in membrane extracts of either blue-irradiated or dark control buds (lanes 1 and 2). By contrast, such phosphorylation is most strongly observed in gels of membranes from the upper internodes—the growing region directly basal to the apical hook; membranes from
With irradiation of either excised tissues or intact seedlings, the level of radioactivity in most of the proteins of other mol wt that are phosphorylated in vitro appears unchanged by blue light. However, one 32P-labeled band at an apparent molecular mass of 84 kD also appears to decrease with prior exposure to blue light, but its tissue distribution differs markedly from the 120 kD phosphorylated band. The level of detectable phosphorylation for this protein is much higher in more basal tissues, in contrast to that of the 120 kD band. The characteristics of this protein have not yet been examined in detail. Note that there are also several apparently tissue-specific phosphorylated proteins observable by this procedure (Fig. 1A), although they do not appear to be responsive to blue light under these conditions.

Fluence-Response Relationships

Two sets of fluence-response data were generated. In the first case, a constant fluence rate of 8 μmol/m²s was used for pulses ranging from 0.1 to 300 s, resulting in fluences of 0.08 μmol/m² to 2.4 × 10³ μmol/m². Several of these experiments included a 5 min waiting period in darkness following the onset of irradiation while in the remaining experiments the tissue segments were homogenized immediately after the light pulse (Fig. 2A). This comparison was made to determine whether a part of the phosphorylation change was the result of a time-dependent, in vivo biochemical dark reaction that might go to completion only during the longer irradiations. In the plants ground immediately after irradiation, the apparent threshold for the response is approximately 0.7 μmol/m² and saturation is about 400 μmol/m² (solid line). In those allowed a 5 min dark period, threshold is near 0.3 μmol/m² and saturation at 160 μmol/m² (broken line). While the sections ground immediately show a slight apparent shift toward lower sensitivity, the difference is within the error of the quantitation procedures. Combining the results from both sets of experiments, we find the threshold for a decrease in measurable phosphorylation at approximately 0.6 μmol/m² while saturation occurs at 250 μmol/m² (Fig. 2A).

In order to test whether the Bunsen-Roscoe law of photochemical equivalence is valid for this system, a fluence-response curve was generated by varying the fluence rate for a constant 30 s and grinding immediately rather than changing the irradiation time with a given fluence rate. These data are shown in Figure 2B. A regression line for these experiments indicates that the threshold occurs around 0.2 μmol/m² and the response becomes saturated at about 160 μmol/m². This result is not significantly different from the other fluence-response curves described above, and therefore it is concluded that reciprocity is valid within the range of fluences tested and the limits of this methodology.

Because of the earlier finding that even long periods of red light irradiation do not significantly affect the level of in vitro phosphorylation (11), red safelights were used instead of green light during harvests and later manipulations. This change in light regime was made to minimize light spillover from the safelights into the photoactive blue portion of the spectrum. A simple set of fluence response curves was obtained with all manipulations performed under either red or green safelights. This experiment confirmed that, in the half-hour harvest

unirradiated segments of this tissue yield a heavy band of radioactivity of 120 kD (lane 3), while those from blue light-exposed sections have essentially no labeled phosphate at the corresponding molecular mass (lane 4). Membranes of more basipetally localized tissue showed progressively lower phosphorylation levels in the dark controls (lanes 5 and 7), but in every case blue light eliminated detectable radioactivity of any protein at 120 kD (lanes 6 and 8). Earlier experiments including tissue from the first (most basal) internode confirm this trend. Root tissues were not included in this set of experiments.

Similar experiments in which irradiations were performed on intact seedlings gave identical results (data not shown). The buds lacked phosphorylation activity in the 120 kD region, and membranes from the growing region of the epicotyls contain the highest dark levels of 32P in a corresponding SDS-PAGE band. Again, membrane extracts from successive zones below the apical hook showed decreasing amounts of radioactivity at 120 kD, but in all tissues there was minimal radioactivity in this region if the plants had been exposed to blue light immediately prior to harvest.
period, there was no discernible difference in the blue light response between experiments conducted under either red or green safelight. Longer periods under dim green could have an effect, however, presumably because the short wavelength tail of the green light source could be absorbed by the photoreceptor.

**Dark Recovery Kinetics**

Although an in vivo saturating pulse of blue light prevents detection of in vitro phosphorylation of a 120 kD protein, the capacity for in vitro phosphorylation at this molecular mass returns if the irradiation is followed by a period of darkness in vivo prior to membrane isolation. Since recovery from saturating blue light irradiations is also found in various physiological systems (see "Discussion"), experiments were performed to determine the extent and kinetics of this recovery process for the phosphorylation response. Three sets of experiments were carried out (Fig. 3); the first used a subsaturating fluence (20 \( \mu \text{mol/m}^2 \); Fig. 3A) which, according to the fluence-response data, reduced the phosphorylation to about 35% of the dark control level, the second a near-saturating fluence (80 \( \mu \text{mol/m}^2 \); Fig. 3B), and the third a supersaturating fluence (3 \( \times 10^3 \) \( \mu \text{mol/m}^2 \); Fig. 3C). The protocol was designed so that the segments for each treatment were incubated for equal periods of time between harvest and homogenization (approximately 95 min) irrespective of dark recovery time.

Following the near-saturating pulse (Fig. 3B), recovery begins between 10 and 20 min after the light pulse, reaches a level within 80% of the dark controls between 45 and 60 min after irradiation, and remains at that level until at least 90 min. Although the same kinetics hold for the first hour of the recovery from a supersaturating pulse (Fig. 3C), there is also

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**Figure 2.** Fluence response relationships for the decrease in the capacity for detectable in vitro phosphorylation. A, Closed symbols and the solid regression line (——) represent results of experiments in which the tissue was ground immediately following irradiation of 8 \( \mu \text{mol/m}^2 \) for varying amounts of time. Experiments in which the segments were allowed to incubate for 5 min from the start of irradiation are indicated by open symbols and the dashed regression line (---). The regression line for the combined data is shown as a dotted line (⋯⋯⋯). The \( r^2 \) values for the regressions are 0.87 for the open symbols, 0.95 for the solid symbols, and 0.86 for all data points. B, Neutral density filters were used to alter the fluence rate of a constant 30 s blue light pulse \( (r^2 = 0.96) \). The similarity of the curves over the full range of fluences indicates that reciprocity is valid within the parameters tested.

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**Figure 3.** Dark recovery of detectable phosphorylation following a blue light exposure. Stem segments were irradiated with a subsaturating pulse of 20 \( \mu \text{mol/m}^2 \) (A), an 80 \( \mu \text{mol/m}^2 \) saturating pulse (B), or a 300 \( \mu \text{mol/m}^2 \) supersaturating pulse (C). The segments were left in darkness for varying lengths of time as indicated, then membranes were extracted and phosphorylated as described in "Materials and Methods."
an overshoot by the 90 min timepoint. During recovery from a subsaturating pulse (Fig. 3A), there is a slightly longer lag period of 20 to 30 min. After the 20 min point the curve is similar to those in Figure 3, B and C. There may be a slight overshoot 60 min after the subsaturating pulse as well.

Changes at the Protein Level

The Coomassie-stained gels of membranes from etiolated pea seedlings exhibit a minor protein band with molecular mass corresponding to that of the phosphorylated band. A blue light pulse sufficient to prevent the in vitro phosphorylation response also caused the disappearance of the 120 kD protein band. The protein band shows identical tissue distribution (Fig. 1B) to that of the phosphorylated protein of the same molecular mass (Fig. 1A). It is not detectable in either the dark or light-exposed buds. In the etiolated apical sections, the difference between dark controls (lane 3) and blue treated (lane 4) is especially striking. As with the phosphorylation, more basal sections of unexposed tissue show decreasing amounts of the protein (lanes 5 and 7), all of which disappears upon exposure to blue light (lanes 6 and 8).

The fluence response requirements for disappearance of the protein band also match those for loss of detectable phosphorylation at that molecular mass; intermediate phosphorylation levels correspond with an incomplete loss of the visible protein band on Coomassie-stained gels (data not shown). Similarly, recovery of in vitro phosphorylatability is matched by a reappearance of the protein band at 120 kD (data not shown). No coincident appearance or increased intensity of other Coomassie-stained bands (which might represent degradation products, polymerization of the protein, or a change in protein mobility caused by a different degree of phosphorylation) have been detected, although such products could be masked by more abundant, comigrating polypeptides.

Irradiation of Isolated Membranes

In vitro exposure of the isolated crude membrane preparation to blue light also affects the phosphorylation of a protein near 120 kD. Contrary to initial expectations, when isolated membranes were irradiated with blue light for 1 min on ice immediately prior to phosphorylation, the 120 kD band became more heavily phosphorylated than the corresponding protein from membranes that were not irradiated in vitro (Fig. 4). While membranes extracted from etiolated seedlings show the characteristic heavy phosphorylation (lane 1), exposure of the same membranes to a blue pulse causes a strong enhancement of the phosphorylation effect (lane 2). Membranes isolated from blue light-irradiated stem sections—which normally do not exhibit a strongly phosphorylated 120 kD band (lane 3)—show a partial return of the capacity for in vitro phosphorylation if exposed to blue light in vitro (lane 4). However, the Coomassie-stained gels do not show a concomitant increase in the protein level at the molecular weight of interest (Coomassie data not shown), although a small change in amount could have escaped detection.

Figure 4. Effect of light given in vitro to isolated membranes prior to phosphorylation. Lane 1 shows the phosphorylation pattern of membranes extracted from totally etiolated stem segments and without exposure to blue light prior to phosphorylation. The arrow indicates the 120 kD phosphorylation band. Lane 2 shows an aliquot of the same membrane preparation which received 60 s of 150 μmol/m2's blue light immediately prior to phosphorylation. Lanes 3 and 4 show membranes from epicotyl segments exposed to 240 μmol/m2 prior to membrane isolation, then given no further light (lane 3) or a second irradiation in vitro (lane 4) as in lane 2. In vitro irradiations were performed in glass culture tubes on ice.

DISCUSSION

The work described above was designed to characterize further the change in detectable phosphorylation initially described by Gallagher et al. (11). A crude tissue distribution study of the phosphorylation reaction indicates that it is primarily the rapidly growing region of the stem that responds to blue light. Since both phototropism (in Cucumis, 10) and growth inhibition (Cucumis, 9; Pisum, 16) are most sensitive to blue light irradiation of the growing region, the change in in vitro phosphorylation could play a role in either of these responses.

The fluence requirements for phototropism and growth inhibition have been reported to differ from one another. Hence, analysis of the fluence-response relationships for the phosphorylation could aid in determining which, if either, of the responses matches these fluence-response measurements. The threshold and saturation determinations shown in Figure 2A are very close to those reported for first positive phototropism in pea epicotyls by Baskin (0.03 μmol/m2 and 300 μmol/m2, respectively; 1). This range is comparable to phototropic fluence-response curves obtained for other tissues (2, 3, 12, 26). On the other hand, Laskowski and Briggs (16) obtained threshold and saturation values for rapid inhibition...
of elongation in red light-grown pea stems more than an order of magnitude higher than those shown in Figure 2A. The pea seedlings used to obtain fluence response measurements for both phototropic curvature and inhibition of growth were grown under dim red light, while those reported here for the phosphorylation response were etiolated. Although preirradiation with red light is known to affect responsiveness to subsequent blue light (e.g., phototropism, 14), fluence response measurements of the change in detectable phosphorylation in membranes from red-grown peas are similar to those from etiolated plants (data not shown). Warpeha and Kaufman (24) studied inhibition of elongation in red-grown plants 24 h after giving a blue light pulse and found a wider range of active fluences than those for the more rapid inhibition of growth (16) or for the observable change in in vitro phosphorylation.

It is clear from experiments with very brief irradiations that the response is quite rapid; even experiments with high-fluence-rate blue pulses of less than 1 s duration followed by immediate (<5 s) grinding in ice-cold buffer gave no indication of a dark reaction between irradiation and grinding of the sections (data not shown). The data presented in Figure 2A suggest that little or no significant difference in fluence response occurs between samples ground at 0 to 4°C immediately after irradiation and those allowed to incubate for 5 min following onset of the blue light exposure. These results imply that any biochemical dark reactions probably are occurring during irradiation and the period of about 5 s between the end of the blue pulse and the start of grinding the sections. Preliminary experiments in which segments were irradiated on ice yielded results not significantly different from those with control segments irradiated at 24°C (data not shown), lending further support to the hypothesis that any biochemical reactions required for the change in detectable phosphorylation are very rapid. In any case, it is apparent that the response occurs well before the onset of either growth inhibition (2-3 min; 16) or phototropism (5–10 min; 1) in Alaska pea and hence could represent an early step along either transduction chain. While kinetics of the response to in vitro irradiation have not yet been done, 60 s of blue light given to the isolated membranes at 4°C immediately before phosphorylation gives a very strong increase in phosphorylation of the 120 kD band (Fig. 4), indicating that the effect is rapid even at low temperatures.

Whereas the level of detectable in vitro phosphorylation of the 120 kD protein was greatly decreased by irradiating the epicotyl sections with blue light, the capacity for phosphorylation gradually returned if the pulse was followed by a period of darkness prior to membrane isolation. This finding is compatible with physiological observations of phototropism in oat and maize coleoptiles in which a saturating unilateral or a bilateral blue pulse was given, followed by varying periods of darkness, and then with a second, normally effective unilateral stimulus (4, 12). The plants were initially insensitive to the second pulse, but gradually recovered their normal photosensitivity over about 40 min. The timing of the return also fits well with a recovery of rapid growth following an inhibitory blue pulse (16). Finally, stomatal guard cells exhibit a similar recovery kinetic for blue-light sensitivity when exposed to a saturating blue light pulse sufficient to induce a maximal opening response (13, 25). The finding that recovery from supersaturating irradiations did not differ significantly from recovery after a barely saturating blue light pulse (at least until 1 h after irradiation, see Fig. 3, B and C) indicates that an excess light stimulus apparently is not stored biochemically at any step between light reception and the control of phosphorylation.

The isolated membranes also react to light given in vitro, although the effect is opposite that expected from results of in vivo irradiations. If it can be demonstrated that the substrate protein and kinase are identical in both the in vivo and in vitro irradiation experiments, the in vitro system will provide a useful approach for further work toward elucidating the role of this reaction and possibly for isolating various biochemical requirements. Since blue light affects detectable phosphorylation not only of intact cells but also of crude membrane preparations, apparently a blue light photoreceptor is present in these isolates and is still able to regulate this specific phosphorylation. In fact, preliminary separations of plasma membranes by aqueous dextran/polyethylene glycol phase partitioning indicate that all the requirements for photosensitivity of the phosphorylation are present in the plasmalemma-enriched fraction (data not shown).

This study should be aided also by our finding that a minor protein component, also at 120 kD, appears and disappears with tissue distribution, kinetics, and fluence-response relationships indistinguishable by eye from those of the phosphorylation response. If this protein can be identified as the phosphorylated species, as seems likely, isolation of the protein, antibody production, and N-terminal sequencing will be invaluable in answering some of the questions presented by this phenomenon.

The light-sensitive change in detectable phosphorylation of a protein with comparable mol wt has been found in the apical 2 to 3 mm of maize coleoptiles (S Gallagher, unpublished results). This finding provides evidence that the mechanism may be conserved in divergent organisms.

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LITERATURE CITED

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