Holophytochrome Assembly

COUPLED ASSAY FOR PHYTOCHROMOBILIN SYNTHASE IN ORGANELLO*

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Utilizing an in vitro coupled assay system, we show that isolated plastids from cucumber cotyledons convert the linear tetrapyrrole biliverdin IXα to the free phytochrome chromophore, phytochromobilin, which assembles with apophtyochrome to yield photoactive holoprotein. The spectral properties of this synthetic phytochrome are indistinguishable from those of the natural photoreceptor. The plastid-dependent biliverdin conversion activity is strongly stimulated by both NADPH and ATP. Substitution of the nonnatural XIIIα isomer of biliverdin for the IXα isomer affords a synthetic holophytochrome adduct with blue-shifted difference spectra. These results, together with experiments using boiled plastids, indicate that phytochromobilin synthesis from biliverdin is enzyme-mediated. Experiments where NADPH (and ATP) levels in intact developing chloroplasts are manipulated by feeding the metabolites 3-phosphoglycerate, dihydroxyacetone phosphate, and glucose 6-phosphate or by illumination with white light, support the hypothesis that the enzyme that accomplishes this conversion, phytochromobilin synthase, is plastid-localized. It is therefore likely that all of the enzymes of the phytochrome chromophore biosynthetic pathway reside in the plastid.

Phytochrome is an important light receptor that regulates many growth and developmental responses in plants (1). The functional photoreceptor is comprised of an apoprotein of approximately 1100 amino acids and a covalently bound linear tetrapyrrole (bilin) chromophore (2). Phytochrome synthesis therefore involves the convergence of two biosynthetic pathways: one for the apoprotein and another for the chromophore. Phytochrome has been shown to regulate its own transcription and, for this reason, the processes involved in the biosynthesis of the apoprotein have been extensively investigated (3, 4). Much less is known about the biochemistry and regulation of phytochrome chromophore synthesis. Knowledge of this biochemical pathway is necessary to fully appreciate how the level of potentially active phytochrome (holophytochrome) is regulated within the cell.

In order to better understand the biosynthetic pathway of the phytochrome chromophore, studies in our laboratory have focused on several questions. What are the intermediates in this biosynthetic pathway? Where in the cell are the enzymes required for phytochrome chromophore synthesis localized? What enzymes or cofactors are needed for holoprotein assembly? Our studies have established that both 5-aminolevulinic acid and biliverdin IXα (BV) are biosynthetic precursors of the phytochrome chromophore (5, 6). More recently, we reported that holophytochrome assembly is autocatalytic and requires only apoprotein and a bilin with an ethylidene functionality in the A-ring, such as the related bilin chromophore phycocyanobilin (PCB) (7, 8). These studies also showed that holoprotein assembly does not require the cofactors ATP or NAD(P)H. Our studies and similar studies on the biosynthesis of PCB in the red alga Cyanidium caldarium (9) support the model for the biosynthetic pathway of the phytochrome chromophore shown in Scheme 1.

This manuscript focuses on phytochromobilin synthase, the penultimate enzyme of the phytochrome chromophore biosynthetic pathway. This enzyme catalyzes the conversion of BV to phytochromobilin, the free pigment that can assemble with apophytochrome to form photoactive holoprotein. Although little is known about this enzymatic conversion in higher plants, it is a reasonable assumption that phytochromobilin synthase is similar to the enzyme BV reductase, which accomplishes the conversion of BV to PCB in the red alga C. caldarium (10–12). In vitro studies using Cyanidium extracts have shown that this conversion is NADPH-dependent (11). In Cyanidium, both BV reductase and heme oxygenase, the enzyme that produces BV from heme, are ferredoxin-dependent (13). It is therefore likely that phytochromobilin synthase is plastid-localized. In the present study, we utilize holophytochrome assembly as a coupled assay for phytochromobilin synthase activity. These experiments demonstrate that phytochromobilin synthesis from BV is mediated by a plastid-localized enzyme system and that the newly synthesized holophytochrome is spectrophotometrically indistinguishable from the natural photoreceptor.

MATERIALS AND METHODS

Reagents

Biliverdin IXα and XIIIα were prepared as described in Elich et al. (6). 4-Amino-5-hexynoic acid (AHA) was a gift from Merrell Dow Pharmaceuticals (Cincinnati, OH). BSA was obtained from Boehringer Mannheim; Hapes from Calbiochem; agar from Difco; polyethyleneimine from Eastman Kodak; EDTA (disodium salt), HCl, and NaOH from Fisher; ethylene glycol from Mallinckrodt; and ultrapure (NH4)2SO4 from Schwarz/Mann. The enzymes glucose-6-phosphate dehydrogenase (specific activity, 500 units/ml) and glutathione reductase.

* The abbreviations used are: BV, biliverdin IXα; AHA, 4-amino-5-hexynoic acid; BSA, bovine serum albumin; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DHAP, dihydroxyacetone phosphate; Hapes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PCB, phycocyanobilin; PFG, 3-phosphoglycerate; TESS, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

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Husked oat seeds (*Avena sativa* L. cv Garry) were imbibed overnight in the dark at 4 °C in the presence of 1 mM AHA in 15 mM Hepes/NaOH buffer, pH 7.4 (14). Seeds were then germinated in complete darkness at 20 °C on 1% agar containing 1 mM AHA in 15 mM Hepes/NaOH, pH 7.4 buffer. Oat seedlings were harvested after 5 days. Cucumber seeds (*Cucumis sativus* L. cv Beit Alpha) were germinated in complete darkness at 30 °C and grown for 7 days. For developing chloroplast preparations, 7-day-old etiolated seedlings were irradiated with bright white light (60-70 pmol m⁻² s⁻¹) in a ratio of 3 to 1 (v/w) and allowed to stir gently for 20 min. After filtration through one layer of cheesecloth and centrifugation at 8000 g for 1 min, the supernatant was recentrifuged at 15000 g for 1 min and centrifuged at 200,000 g for 15 min. The concentration required for the attachment assay.

**Plastid Isolation**

Pastists were isolated according to the procedure of Fuesler et al. (15). Cucumber cotyledons were harvested and homogenized at 4 °C in a precooled pestle and mortar with cold homogenization medium in a ratio of 4 to 1 (v/w). This medium contained 20 mM Hepes buffer, 1% (w/v) BSA, 10 mM Hepes/NaOH buffer (pH 7.7), 500 mM sorbitol, 1 mM MgCl₂, 1 mM EDTA (free acid), 1 mM EDTA (disodium salt), 5 mM cysteine, and 0.2% (w/v) BSA. The homogenate was filtered through four layers of cheesecloth and centrifuged at 8000 g for 1 min. The pellet containing intact pastists were resuspended in 20 mM TES, 10 mM Hepes-NaOH buffer (pH 7.7) containing 500 mM sorbitol, 1 mM PMSF, 0.5 mM diithiothreitol, and 2 μM leupeptin.

**Coupled Assay For Phytochromobilin Synthase**

Two variations of the phytochromobilin synthase assay were used for these studies. **Procedure A**—In this procedure, plastid preparations (5-10 mg of protein/ml) were incubated for 30 min at 28 °C in the dark (unless otherwise specified) in 700 μl of plastid incubation medium (20 mM TES, 10 mM Hepes, NaOH buffer (pH 7.7) containing 500 mM sorbitol, 1 mM PMSF, 0.5 mM diithiothreitol, 2 μM leupeptin, 10 μM biliverdin IXα, and other additions as specified under "Results"). The reaction was stopped by removal of the plastids by centrifugation at 100 × g for 1 min. To 400 μl of the resulting supernatant was added 300 μl of the phytochrome apoprotein preparation, which consisted of 5.0-15.0 pg/ml total phytochrome polypeptides containing 2.5-4.0 pg/ml holophytochrome. This mixture was then incubated for 30 min at 28 °C in the dark. The samples were cooled on ice and clarified by centrifugation at 200,000 × g for 15 min. The concentration of spectrally active phytochrome was determined. Where specified, light treatments consisted of bright white light (from two fluorescent strip lights, 120 μmol m⁻² s⁻¹ photosynthetically active radiation) given for the duration of the first incubation only. In these experiments, a NADPH-scavenging system was employed. This consisted of 10 μM oxidized glutathione and 0.1 units/ml glutathione reductase.

**Procedure B**—In this procedure, the phytochrome apoprotein was present in the same incubation medium as the plastids. Otherwise, the initial incubation was performed using conditions identical to those for Procedure A. After incubation, the reaction was stopped by transfer to 4 °C and centrifugation at 1000 rpm (100 × g) for 1 min in a benchtop microcentrifuge to pellet the plastids. The supernatant was clarified at 200,000 × g for 15 min, and the concentration of spectrally active phytochrome was determined.

**Spectrophotometric Phytochrome Assay**

Clariied samples were assayed for phytochrome as described previously (5), except that red and far-red irradiation times were extended to 200 s. The concentration of phytochrome in μg/ml was calculated using the molar absorption coefficient for P₇ for 688 nm of 1.32 × 10⁴ liters mol⁻¹ cm⁻¹ (16) and a molecular mass of 124 kDa.

**Other Procedures**

Chlorophyll was determined by the method of Arnon (17). Protein was determined using the BCA protein assay (Pierce Chemical Co.) using BSA as a standard.
accomplishes this conversion. A coupled holophytochrome assembly assay system was developed for this purpose. Plastids from cucumber cotyledons were chosen for these experiments because tetrapyrrole metabolism is relatively well characterized in this tissue (9, 18, 19). Both etioplasts and developing chloroplast preparations were examined for their ability to convert BV to the free phytchrome chromophore, phytochromobilin, in the presence or absence of added cofactors. Phytochromobilin synthesis was determined by measuring accumulation of photoactive holophytochrome following addition of apophytochrome to the incubation media. The apophytochrome preparations used for these experiments consisted of soluble protein extracts from etiolated oat seedlings in the presence of AHA, a potent inhibitor of tetrapyrrole synthesis in plants (14).

The standard assay protocol employed for these studies involved preincubation of plastids with BV, in the presence and absence of added cofactors, followed by removal of the organellar material prior to the addition of apophytochrome (Procedure A under “Materials and Methods”). These experiments clearly indicate that etioplast preparations contain the necessary activity for the conversion of BV to a bilin that can assemble with apophytochrome to form photactive holophytochrome. Fig. 1A shows the difference spectra of photoactive phytchrome produced by incubation of apophytochrome with the supernatant from the initial incubation of BV and etioplasts. The level of BV in the absence of BV is indicated by the difference spectrum shown with the dotted line and is equal to the amount of photoactive phytochrome added to the assay medium. AHA treatment does not completely inhibit phytchrome chromophore synthesis; therefore, such extracts contain low levels of photoactive phytochrome (14). When BV was added, a 30–50% increase in the amount of photoactive holophytochrome was typically observed (see Fig. 1A, dashed line). An additional stimulation in holophytochrome synthesis was detected when a NADPH-regenerating system was also present in the assay medium (Fig. 1A, solid line). Since plastids incubated with NADPH alone did not yield any measurable synthesis of photoactive phytchrome (data not shown), these results show that BV is required for the synthesis of new phytchrome holoprotein. Immunoblot analyses confirmed that there was little or no proteolysis of phytchrome during the incubation (data not shown).

Fig. 1B (solid line) shows the difference spectrum of the newly synthesized phytchrome holoprotein. This was obtained by subtraction of the control difference spectrum (i.e. the difference spectrum obtained when BV was omitted from the assay mixture) from that obtained following incubation of plastids with BV and a NADPH-regenerating system. The difference spectrum of the newly synthesized holophytochrome exhibits a difference maximum at 668 nm, a difference minimum at 720 nm, an isosbestic wavelength at 689 nm, and a \( \Delta A_{\text{max}}/\Delta A_{\text{min}} \) ratio of 1.06, which are indistinguishable from those of the native photoreceptor isolated from oats (16). An identical result was observed when greening plastid preparations were used (data not shown). These results clearly show that these plastid preparations contain the enzyme system required for conversion of BV to the natural free phytchrome chromophore, phytochromobilin.

Owing to the stimulatory effect of NADPH on the BV conversion activity of plastid preparations, experiments were also performed to test the relative effectiveness of NADPH and other cofactors on the coupled synthesis of holophytochrome from BV. For comparative purposes, both etioplast and developing (i.e. partially greened) chloroplast prepara-
Table I

| Phycocyanin holoprotein | Etioplasts | Developing chloroplasts |
|-------------------------|-----------|-------------------------|
| Experiment | 1 | 2 | Increase | Experiment | 1 | 2 | Increase |
| µg/ml | µg/ml | % | µg/ml | µg/ml | % |
| −BV | 2.8 | 2.7 | 3.2 | 2.8 | 2.7 | 3.2 |
| +BV (10 µM) | 3.7 | 4.9 | 32.3 | 3.3 | 3.8 | 20.5 |
| +BV + NADPH | 3.9 | 5.4 | 42.6 | 3.6 | 4.7 | 40.1 |
| (1 mM) | | | | | | |
| +BV + NADH | 4.0 | 5.9 | 51.2 | 4.1 | 4.9 | 52.5 |
| (1 mM) | | | | | | |
| +BV + ATP | 5.8 | 7.4 | 103.6 | 5.6 | 5.6 | 91.2 |
| (1 mM) | | | | | | |
| +BV + NADPH | 6.4 | 8.6 | 130.5 | 6.2 | 6.7 | 119.5 |
| +BV + NADPH* | 2.8 | ND | * | | | |
| + heat | | | | | | |

* Mean percent increase of newly synthesized phytochrome holoprotein over the −BV control.
+ Added as a regenerating system containing final concentrations of 1.2 mM NADP*, 9.3 mM glucose 6-phosphate, and 1.5 units/ml glucose-6-phosphate dehydrogenase.
+ From a single different experiment.
+ Not determined.

Table II

| Phytochrome holoprotein increase* | % |
|----------------------------------|---|
| Procedure A | |
| −BV | 0.0 |
| +BV (10 µM) | 55.8 ± 11.7 (6) |
| +BV + NADPH | 129.5 ± 11.2 (9) |
| Procedure B | |
| −BV | 0.0 |
| +BV (10 µM) | 36.4 ± 9.9 (4) |
| +BV + NADPH | 92.8 ± 10.5 (7) |

* Percent increase of newly synthesized phytochrome holoprotein over the −BV control. Values are expressed as mean ± standard error of the mean, and the numbers in parentheses indicate the number of experiments sampled.
+ Apophytochrome incubated with supernatant only. See “Materials and Methods.”
+ Added as a regenerating system containing final concentrations of 1.2 mM NADP*, 9.3 mM glucose 6-phosphate, and 1.5 units/ml glucose-6-phosphate dehydrogenase.
+ Apophytochrome and etioplasts co-incubated. See “Materials and Methods.”

The experiments described above utilized both assay protocols (Procedure A) in which the plastid organelles were removed prior to apophytochrome addition. To verify that the plastid-dependent phytochromobilin synthesis was enzyme-mediated, another experiment was performed in which the plastids were boiled prior to the assay. Boiled plastids did not support new synthesis of holophytochrome even if NADPH was added to the incubation medium (Table I).

The results described above utilized an assay protocol (Procedure A) in which the plastid organelles are removed prior to apophytochrome addition. To verify that the plastid-dependent phytochromobilin synthesis was enzyme-mediated, another experiment was performed in which the plastids were boiled prior to the assay. Boiled plastids did not support new synthesis of holophytochrome even if NADPH was added to the incubation medium (Procedure B). A direct comparison of the efficiency of the two procedures is shown in Table II. These results show that there is a significant increase in the amount of holophytochrome assembly using Procedure B as compared with Procedure A. In fact, a direct comparison under conditions where apophytochrome is not limiting (see Table II, +BV without added NADPH) indicates that the amount of holophytochrome produced by Procedure A is greater than that utilizing Procedure B. These results show that holophytochrome assembly does not require a direct interaction between plastids and apophytochrome and that Procedure B offers no experimental advantage over Procedure A.

In order to further optimize the conditions needed for coupled holophytochrome assembly from BV, the concentration dependence for BV was determined. The results of these experiments are shown in Fig. 2. Under the assay conditions used, 10 µM BV proved to be saturating with half-maximal synthesis occurring at 0.5 µM BV. For this reason, 10 µM BV was routinely included in standard assay mixtures to ensure that BV substrate was not limiting.

Previously we showed that in vivo feeding of the nonnatural BV XIIα isomer to AHA-grown oat seedlings led to the formation of a photoactive phytochrome with altered spectral properties (6). This result implied that BV XIIα was converted to an isomer of phytochromobilin that could assemble with apophytochrome in vivo. An experiment was therefore performed to determine whether plastid preparations can metabolize BV XIIα to a bilin that can assemble with apophytochrome. Fig. 1B (dashed line) shows the difference spectrum of the holophytochrome species produced when the nonnatural BV XIIα isomer was substituted for the natural IXα isomer in our coupled assay system. The difference spectrum of the BV XIIα-derived holophytochrome exhibits a difference maximum at 660 nm, a difference minimum at 714 nm, an isosbestic wavelength at 679 nm and a ΔΔmax/ΔΔmin ratio of 0.90. This unusual difference spectrum is similar with that observed for the holophytochrome species derived from BV XIIα in vivo (6).

Phytochromobilin Synthase Is Localized in the Plastid—In order to address whether phytochromobilin synthase is plastid-localized, experiments were performed using intact etioplast preparations enriched by centrifugation through Percoll, according to the method of Fuesler et al. (15). If etioplasts were extensively washed to remove traces of Percoll, similar levels of phytochromobilin synthase activity were found when assay Procedure A (see “Materials and Methods”) in the

Fig. 2. The effect of BV concentration on the synthesis of new phytochrome holoprotein. Cucumber etioplasts were incubated with different BV IXα concentrations using the assay described in Procedure B (see “Materials and Methods”). Assays also included the NADPH-regenerating system containing final concentrations of 1.2 mM NADP*, 9.3 mM glucose 6-phosphate, and 1.5 units/ml glucose-6-phosphate dehydrogenase. Results are from a single experiment.
The effect of Calvin-Benson cycle metabolites on the new synthesis of phytochrome holoprotein in darkness by plastid preparations isolated from cucumber cotyledons

| Phytochrome holoprotein | Etioplasts | Developing chloroplasts |
|-------------------------|------------|------------------------|
|                         | Experiment | Increase | Experiment | Increase |
| 1 | 2 | μg/ml | % | 1 | 2 | μg/ml | % |
| -BV | 2.8 | 3.7 | 2.7 | 3.2 |
| +BV (10 μM) | 3.7 | 4.9 | 32.3 | 3.3 | 3.8 | 20.5 |
| +BV + PGA (1 mM) | 3.2 | 4.9 | 12.6 | 2.8 | 3.6 | 8.1 |
| +BV + DHAP (1 mM) | 6.0 | 7.5 | 108.5 | 5.4 | 5.3 | 82.8 |

* Mean percent increase of newly synthesized phytochrome holoprotein over the -BV control.

The effect of light on the new synthesis of phytochrome holoprotein in greening chloroplast preparations isolated from cucumber cotyledons

| Phytochrome holoprotein | Experiment | Increase |
|-------------------------|------------|----------|
|                         | 1 | 2 | 3 |
| -BV, dark | 3.6 | 2.8 | 2.5 |
| +BV (10 μM), dark | 4.8 | 4.5 | 3.3 | 42.0 |
| +BV, light | 5.6 | 4.6 | 3.7 | 65.2 |
| +BV + DCMU (5 μM), light | 4.3 | 3.8 | 3.1 | 26.4 |
| +PCB (2 μM), dark | 8.3 | 6.7 | 6.3 | 140.6 |
| +PCB, light | 4.3 | 6.1 | 3.3 | 56.4 |

* Mean percent increase of newly synthesized phytochrome holoprotein over the -BV control.

plastid dilution experiments were performed to provide estimates of the specific activities of phytochromobilin synthase in preparations of both etioplasts and developing chloroplasts. Fig. 3 shows the dependence of plastid protein on the yield of phytochromobilin from BV. In this experiment, the NADPH-regenerating system was present in the assay medium to maximize the yield of phytochromobilin synthesis, and the incubations were performed in darkness. Based on this data, a specific activity of 1.1 pmol of phytochromobilin produced/mg of plastid protein/min was determined for phytochromobilin synthase in etioplasts. For developing chloroplasts, a specific activity of 0.4 pmol of phytochromobilin produced/mg of plastid protein/min (or 11.6 pmol of phytochromobilin produced/mg of chlorophyll/min) was determined similarly. These values represent minimum estimates for the specific activities because our assay measures coupled assembly of phytochrome rather than the production of free phytochromobilin. As was shown in an earlier study, the relationship of phycocyanobilin concentration to phytochrome assembly is not stoichiometric under similar experimental conditions (7). Calculation of a true specific activity will therefore require direct measurement of the rate of phytochromobilin synthesis, which is beyond the scope of the present study.

**TABLE IV**

| Phytochrome holoprotein | Experiment | Increase |
|-------------------------|------------|----------|
|                         | 1 | 2 | 3 |
| -BV, dark | 3.6 | 2.8 | 2.5 |
| +BV (10 μM), dark | 4.8 | 4.5 | 3.3 | 42.0 |
| +BV, light | 5.6 | 4.6 | 3.7 | 65.2 |
| +BV + DCMU (5 μM), light | 4.3 | 3.8 | 3.1 | 26.4 |
| +PCB (2 μM), dark | 8.3 | 6.7 | 6.3 | 140.6 |
| +PCB, light | 4.3 | 6.1 | 3.3 | 56.4 |

* Mean percent increase of newly synthesized phytochrome holoprotein over the -BV control.

**DISCUSSION**

When BV IXα is incubated with a plastid preparation from cucumber cotyledons, it is enzymatically converted to a bilin...
that can assemble with phytochrome apoprotein in vitro. This newly synthesized holoprotein has a difference spectrum that is indistinguishable from that of the native phytochrome holoprotein. We therefore conclude that the bilin synthesized in organello is the natural free phytochrome chromophore phytochromobilin, and for this reason, we name the enzyme that accomplishes this conversion phytochromobilin synthase. In view of the ability of the structurally similar pigment PCB to assemble with apophytochrome and form photoactive holoprotein (7, 8), the chemical structure of the phytochromobilin synthesized in this system is probably that proposed by Rudiger et al. (20) (see Scheme 1). This conclusion remains to be experimentally verified, however. Owing to the high phytochromobilin synthase activity in cucumber plastid preparations, experiments to isolate and chemically characterize phytochromobilin are now technically feasible.

The synthesis of phytochromobilin from BV requires a twoelectron reduction in the A-ring, which is analogous to the four-electron reduction of BV to PCB in the red alga C. caldarium by the NADPH-dependent enzyme BV reductase (10, 11). It is therefore reasonable that phytochromobilin synthase in higher plants is similar to the red algal BV reductase. In this study, we show that phytochromobilin synthase activity is detectable in both etioplast and developing chloroplast preparations in the absence of added cofactors. We propose that this cofactor-independent activity reflects the presence of intact organelles that contain endogenous cofactors. This interpretation is supported by the observation that the presence of the NADPH-scavenging system, oxidized glutathione and glutathione reductase, has no effect on this cofactor-independent activity. Addition of an NADPH-regenerating system, however, strongly enhances phytochromobilin synthesis from BV. This suggests that reducing power provided by a reduced pyridine nucleotide is required for phytochromobilin synthase activity in organello. This is in agreement with the NADPH dependence of the red algal BV reductase system (11). Paradoxically, phytochromobilin synthesis in cucumber plastid preparations is more strongly stimulated by ATP than by the reduced pyridine nucleotides NADPH and NADH. We believe that the stimulation by ATP is due to the generation of phosphorylated substrates, such as glucose 6-phosphate, which are utilized by plastid-localized enzymes of the oxidative pentose phosphate pathway. ATP-dependent stimulation of the NADPH-dependent enzyme, magnesium protoporphyrin IX monomethyl ester (oxidative) cyclase, in the same plastid system has been similarly rationalized (21). In support of this conclusion, we found that glucose 6-phosphate alone supports high rates of phytochromobilin synthesis. Glucose 6-phosphate also has been shown to drive nitrite reductase in isolated pea root plastids (22). Taken together, our cofactor dependence experiments imply that ATP and glucose 6-phosphate are more readily assimilated by plastids than are reduced pyridine nucleotides. In this regard, the occurrence of specific translocators for both ATP and glucose 6-phosphate in plastid envelope membranes from nonphotosynthetic tissue has been well documented (23). In summary, we conclude that the cofactor dependence of phytochromobilin synthase reported here is consistent with that of the red algal BV reductase.

In the above discussion, we have stressed the potential role of ATP and/or glucose 6-phosphate as a source of reducing power. It is also possible that these cofactors may facilitate holophytochrome assembly from phytochromobilin. We previously demonstrated that no cofactors are required for ligation of PCB to phytochrome apoprotein from oat (7, 8). For this reason, it is unlikely that any cofactor would be needed for holophytochrome assembly with the natural phytochrome chromophore. Since our experiments suggest that BV must enter and phytochromobilin must leave the plastid, cofactors may stimulate this bilin pigment trafficking across the plastid envelope. This raises the possibility that regulatory mechanisms may exist for bilin trafficking between the plastid and cytosol, which appears to be the site of holophytochrome assembly in vivo. In view of the amphipathic nature of these pigments, however, passive diffusion across the plastid envelope may be the mechanism of pigment transport. In vivo feeding experiments have shown that the bile pigments BV and PCB enter plant cells, presumably by passive diffusion across the plasma membrane (5, 6).

Experiments to manipulate endogenous NADPH levels by addition of Calvin-Benson cycle intermediates have provided strong support for the hypothesis that phytochromobilin synthase is plastid-localized. In these experiments, we chose the metabolites, PGA and DHAP, to alter endogenous NADPH levels. These studies showed that PGA inhibited phytochromobilin synthase activity, whereas DHAP stimulated this enzyme. These results are consistent with the respective ability of PGA or DHAP to consume or produce NADPH via metabolism by the plastid-localized enzymes, glycerate kinase and glyceraldehyde-3-phosphate dehydrogenase. Moreover, DHAP proved stimulatory even in the presence of oxidized glutathione and glutathione reductase, which should scavenge any NADPH released to the incubation medium. These results confirm that the reducing power is generated within the same organelle compartment (i.e. the plastid) in which phytochromobilin is synthesized.

Parallel experiments with light also support the hypothesis that phytochromobilin synthase is plastid-localized. Although light stimulation is less pronounced than the effect of the metabolites described above, it is reproducible and can be reversed by treatment with the photosynthetic electron transport inhibitor DCMU. We believe that the photosynthetic effect is most likely due to light-driven NADPH (and ATP) synthesis. Other interpretations, such as light activation of phytochromobilin synthase itself, cannot however be dismissed a priori. These results clearly show that phytochromobilin synthase activity is influenced by changes in plastid metabolism and are, therefore, consistent with the conclusion that this enzyme is plastid-localized. Interestingly, our experiments revealed that incubation of the phytochrome chromophore analog PCB with plastids in the light strongly inhibits its subsequent holophytochrome assembly. This light effect is plastid-dependent, since irradiation of PCB in the absence of plastids does not reduce holophytochrome assembly. Phytochromobilin attachment is therefore likely to be inhibited in the same light-dependent manner. In view of these observations, light-driven phytochromobilin synthesis may actually be much greater than that detected by our coupled assay system. Indeed, if DCMU is used to block new NADPH synthesis, then the level of new holoprotein synthesized in the light is lower than the dark control. This finding also supports the idea that there are competing reactions in the light that limit phytochromobilin availability, including the possibility that the phytochrome chromophore is specifically degraded in the light.

In summary, our experiments strongly support the conclusion that phytochromobilin synthase is localized in the plastids of higher plants. The observation that phytochromobilin synthase activity is retained in highly enriched etioplast preparations purified by a Percoll step gradient also is consistent with this conclusion. Our results do not, however, rule out the possibility that this enzyme is also present in another cellular
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compartment (e.g. mitochondria or cytosol) as well. Final confirmation of the subcellular localization of phytochromobilin synthase must await immunolocalization studies, when antibodies to this enzyme become available.

When etiolated plants are transferred to the light, there is a rapid reduction in expression of the phytochrome apoprotein (24). It might therefore be expected that under these conditions, less chlorophyll would be made available by the cell. In addition to the possible effect of light on phytochromobilin stability described above, preliminary determinations of the specific activity of the phytochromobilin synthase have shown that in etioplasts the specific activity is three times greater than in developing chloroplasts isolated from cucumbers exposed to 20 h of white light. This reduction in specific activity is partly due to the increase in protein synthesis in developing chloroplasts, but is nevertheless two times lower than etioplasts on a fresh weight basis.

Our studies have also addressed the question of whether an interaction between the phytochrome apoprotein and the plastid would facilitate chromophore attachment. Comparative experiments with two variations of the coupled assay have shown that there is no requirement for the phytochrome apoprotein to interact with the plastid in order to receive a chromophore. Nor is there an increase in the efficiency of holophytochrome assembly under conditions where phytochromobilin production is limiting. These results suggest that the phytochrome chromophore is released into the cytoplasm in vivo where it assembles with the apoprotein. How holophytochrome assembly is regulated in vivo is not known. Since it is well established that plant cells accumulate apophytochrome under conditions where the synthesis of the chromophore has been inhibited (5, 25, 26), it is conceivable that a significant apoprotein pool might be present under normal conditions. Regulation of phytochrome chromophore synthesis and/or transport through the plastid envelope could therefore prove to be important in determining the concentration of active phytochrome (phytochrome holoprotein) in the cell.

In this paper, we have demonstrated that BV can be enzymatically converted to phytochromobilin in both etioplasts and developing chloroplasts isolated from cucumber cotyledons. Isolated plastids will therefore provide an ideal starting material for the purification of phytochromobilin synthase enzyme. Preliminary experiments indicate that plastid preparations from oat seedlings are also able to convert BV to phytochromobilin, and the occurrence and properties of this enzyme in other plant species are currently under examination. It will be of particular interest to determine whether this enzyme is present in lower plant species where phytochrome or phytochrome mediated responses have not been detected. The coupled assembly assay described in this paper should also permit the determination of the other intermediates in the biosynthetic pathway of the phytochrome chromophore. Preliminary experiments have already shown that phytochromobilin can be synthesized from ALA, indicating that the entire phytochrome chromophore biosynthetic pathway is present in the plastid. An understanding of the biochemistry of the enzymes of this pathway may allow us to produce plants in which the biosynthesis of the chromophore is completely inhibited. Such plants, which lack holophytochrome, would provide valuable information on the role of phytochrome in vivo. For this reason, the substrate and inhibitor specificity of phytochromobilin synthase is currently under investigation. Knowledge of the biosynthetic pathway of the phytochrome chromophore will also facilitate characterization of higher plant mutants in which immunodetectable phytochrome is present in excess of the photoreversible holoprotein (27, 28). Such mutants may be impaired in their ability to synthesize phytochromobilin. Our studies also demonstrate that the BV isomer, BV XIIIa, can be converted to a lightable isomer of phytochromobilin. By feeding modified BVs and radiolabeled BV to the plastids, a wide range of novel or radiolabeled phytochrome chromophores can now be synthesized. In view of the observation that phytochrome apoprotein expressed in both yeast and Escherichia coli is able to ligate phytochromobilin synthesized in plastid preparations to give the native holoprotein (29), a large variety of synthetic holophytochromes with altered apoproteins and/or chromophores can be prepared. This capability will facilitate analyses of the structure and function of this important photoreceptor molecule.

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