Phytochrome Assembly

THE STRUCTURE AND BIOLOGICAL ACTIVITY OF 2(R),3(E)-PHYTOCHROMOBILIN DERIVED FROM PHYCOBILIPROTEINS*

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The unicellular rhodophyte, Porphyridium cruentum, and the filamentous cyanobacterium, Calothrix sp. PCC 7601, contain phycobiliproteins that have covalently bound phycobilin chromophores. Overnight incubation of solvent-extracted cells at 40°C with methanol liberates free phycobilins that are derived from the protein-bound bilins by methanolytic cleavage of the thioether linkages between bilin and apoprotein. Two of the free bilins were identified as 3(E)-phycocyanobilin and 3(E)-phycoerythrobilin by comparative spectrophotometry and high pressure liquid chromatography. Methanolyis also yields a third bilin free acid whose absorption and 1H NMR spectra support the assignment of the 3(E)-phytochromobilin structure. This novel bilin is the major pigment isolated from cells that are pre-extracted with acetone-containing solvents. Since phytochrome- or phytochromobilin-containing proteins are not present in either organism, the 3(E)-phytochromobilin must arise by oxidation of phycobilin chromophores. This pigment is not obtained by similar treatment of a cyanobacterium and a rhodophyte that lack phycocyanin. Therefore, 3(E)-phytochromobilin appears to be derived from phycoerythrobilin-containing proteins. Comparative CD spectroscopy of 3(E)-phytochromobilin and 3(E)-phycocyanobilin suggests that the two bilins share the R stereoschemistry at the 2-position in the reduced pyrrole ring. Incubation of 2(R),3(E)-phytochromobilin with recombinant oat apophytochrome yields a covalent bilin adduct that is photoactive and spectrally indistinguishable from native oat phytochrome isolated from etiolated seedlings. These results establish that the bilinprotein-derived 2(R),3(E)-phytochromobilin is a biologically active phytochrome chromophore precursor.

The phytochrome chromophore bears a close structural resemblance to the chromophores of phycocyanin and phycoerythrin, the phycobiliprotein light-harvesting pigments of rhodophyte and cryptophyte algae and cyanobacteria (Rudiger et al., 1980; Lagarias et al., 1979; Lagarias and Rapoport, 1980; Crespi et al., 1967; Cole et al., 1967; Chapman et al., 1967; Crespi and Katz, 1969). All of these proteins contain linear tetrapyrrole prosthetic groups (Fig. 1) that, in their functional state, are covalently linked to specific cysteine residues of the proteins. In addition to having similar structures, these pigments share a common biosynthetic pathway and are derived from protoporphyrin IX α (Beale and Weinstein, 1991).

Cleavage of the thioether bilin-protein linkages by heating phycobiliproteins with methanol or strong acids liberates free bilins that have been characterized by spectroscopy and total synthesis (Cole et al., 1967; Crespi et al., 1967; Chapman et al., 1967; Crespi and Katz, 1969; Gossauer and Hirsch, 1974; Gossauer and Weller, 1978; Schram and Kroes, 1971). Owing to the low abundance of phytochrome in plant tissue and to the chemical reactivity of the released bilin chromophore, the isolation of phytochromobilin has been considerably more difficult. Although both methanolyis and HBr-trifluoroacetic acid treatment liberate bilin pigments from phytochrome, only trace amounts of phytochromobilin have been obtained (Siegelman et al., 1966; Kroes, 1970; Rüdiger et al., 1980). The major pigment isolated from HBr-trifluoroacetic acid treatment of phytochrome was a methanol adduct of phytochromobilin (Rüdiger et al., 1980).

Cleaved bilin free acids have proven to be useful reagents for the study of phycobiliprotein chromophore biosynthesis as well as the biosynthesis of the free phytochrome chromophore and its assembly with the phytochrome apoprotein. For example, the intermediacy of 3(Z)-phycoerythrobilin and 3(Z)-phycocyanobilin in the enzymatic conversion of biliverdin IX α to 3(E)-phytochromobilin in Cyanidium caldarium has been established by using the methanolytically released free bilins as standards and enzyme substrates (Beale and Cornejo, 1991b). Because phytochromobilin has not been readily available, the intermediacy of this pigment in phytochrome biosynthesis has been inferred from the ability of phycocyanobilin to substitute for the natural chromophore precursor both in vivo (Elich et al., 1989) and in vitro (Elich and Lagarias, 1989; Lagarias and Lagarias, 1989; Wahleithner et al., 1991; Deforce et al., 1991). The latter studies have established that the formation of photochemically active holophytochrome proceeds spontaneously in the absence of added enzymes or cofactors. Using holophytochrome assembly as an assay, Terry and Lagarias (1991) have recently presented evidence for phytochromobilin synthase, the enzyme that catalyzes conversion of biliverdin IX α to the phytochrome chromophore precursor, in higher plant plastid preparations. By contrast to the phycocyanobilin-apophytochrome adduct, which is spectrally blue-shifted, the newly synthesized holophytochrome species is spectrophotometrically indistinguishable from native phytochrome isolated from plant tissue. On the basis of this evidence, it was concluded that the plastid enzyme produces the natural phytochrome chromophore pre-
The abbreviations used are: TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

**EXPERIMENTAL PROCEDURES**

**Growth of Cells—** Axenic liquid suspension cultures of *Porphyridium cruentum* UTEX 637 and *C. coeladarium* strain CPD were grown in liquid medium as described previously (Beale and Chen, 1983; Beale and Cornejo, 1991a, 1991b), with illumination (32 μE m⁻² s⁻¹) provided by equal numbers of cool-white and red fluorescent tubes. Use of the synthetic bilins in biosynthetic studies is problematic because they are racemic, whereas the natural bilins are likely to be chiral. Also, saponification of the esters is expected to afford low yields of the bilin free acids because of isomerization of the ethylidene-bearing reduced pyrrole ring to an ethylpyrrole (Cole et al., 1967).

During ongoing studies of phycobilin biosynthesis in algae, a new pigment appeared under certain extraction conditions. This pigment has now been identified as 2(R),3(E)-phytochromobilin. Although the occurrence of this pigment in the algal extracts is probably artificial and has no biosynthetic significance, it has provided a source of sufficient phytochromobilin for testing as a substrate for phytochrome reconstitution experiments. Results reported here show that this pigment can spontaneously assemble with recombinant oat apophytochrome to form photoactive holophytochrome that contains a covalently bound chromophore and is spectrally indistinguishable from native oat phytochrome.

**Phytochromobilin Structure and Activity**

![Phytochromobilin Structures](image)

**FIG. 1. Bilin structures.** Structures of biliverdin IXα, the 3(E) isomers of free phycocyanobilin and phycoerythrobilin, the 3(E) and 3(Z) isomers of free phytochromobilin, and the phytochromobilin undecapeptide from oat phytochrome discussed in the text. The conventional carbon numbering system used in the text is shown for biliverdin IXα.
those of all bilins previously characterized in methanolysis of racemic 3(E)-phytochromobilin dimethyl ester dissolved in very close to those reported for chemically synthesized racemic 3(E)-phytochromobilin. The spectrum had maxima at 386 and 700 nm, and the absorption maxima for phycocyanobilin and phytochromobilin, respectively. After incubation for 30 min at 25°C, reaction mixtures were clarified by ultracentrifugation for 15 min at 200,000 x g and assayed for holophytocrome as described below.

Holoophytocrome Assays—Phytochrome difference spectra at 5°C were obtained using HP 8450A and Aviv 14DS spectrophotometers as described previously (Wahleithner et al., 1991). Incubations were initiated by adding 5 μl of bilin stock solution in dimethyl sulfoxide to 600 μl of incubation medium containing 25 mM Tris-HCl (pH 7.8), 2 mM EDTA, 2 mM phenylmethanesulfonyl fluoride, 1 mM diithiothreitol, 25% (v/v) ethylene glycol, and 9 μg of apophytocrome. The protein concentration in the final incubation mixture was 2.5 mg/ml. Final bilin concentrations were 4 μM and 1.7 μM for phycocyanobilin and phytochromobilin, respectively. After incubation for 30 min at 25°C, reaction mixtures were clarified by ultracentrifugation for 15 min at 200,000 x g and assayed for holophytocrome as described below.

RESULTS

P. cruentum Phycobiliprotein Methanolysis Products—In the initial experiments, methods for extracting free pigments and other soluble materials from the cells before methanolysis were adapted from those used previously for C. caldarium (Beale and Cornejo, 1991b). For C. caldarium, it was necessary to pre-extract the cells with dimethyl sulfoxide to disrupt the cell membranes. After the dimethyl sulfoxide treatment, methanol was used to remove dimethyl sulfoxide and complete the cell extraction before methanolysis. In early attempts to apply this procedure to P. cruentum cells, it was found that the cells did not sediment in dimethyl sulfoxide, but that sedimentation could be achieved if the dimethyl sulfoxide solution was diluted 8-fold with acetone. The procedure that was adopted was to extract the cells with dimethyl sulfoxide, dilute the solution 8-fold with acetone, and then extract four more times with dimethyl sulfoxide/acetone (1:8, v/v) and finally three times with methanol.

After extraction by the above procedure, the cells were methanolized. The bilins obtained were purified by solvent partitioning and DEAE-Sepharose chromatography and separated by reverse-phase HPLC. Three prominent peaks were apparent in the elution profile (Fig. 2). The middle and late-eluting pigments were identified as 3(E)-phycocyanobilin and 3(E)-phycoerythrobilin, respectively, by comparative HPLC and spectrophotometry (Table I). The early-eluting peak had an elution position and absorption maxima that differed from those of all bilins previously characterized in methanolysis extracts and enzyme incubations (Table I). Identification of the early-eluting pigment is described below.

Absorption Spectroscopy of the Early Eluting Pigment—The visible absorption spectrum of the HPLC-purified pigment was recorded in methanol/36% (w/v) aqueous HCl (48:2, v/v). The spectrum had maxima at 386 and 708 nm, and the peak height ratio was 1.67 (Fig. 3a; Table I). These values are very close to those reported for chemically synthesized racemic 3(E)-phytochromobilin dimethyl ester dissolved in methanol/HCl (49:1, v/v) (Weller and Gossauer, 1980). The synthetic pigment has absorption maxima at 386 and 707 nm and a peak height ratio of 1.70 (Weller and Gossauer, 1980). Differences between the two spectra are attributed to the different esterification states of the two bilins and/or slight differences in the solvent composition. On the basis of spectral similarity, the early-eluting pigment was provisionally identified as phycocyanobilin. It could not be determined from the absorption spectrum whether the pigment more closely resembles 3(E)- or 3(Z)-phytochromobilin.

1H NMR Spectroscopy of the Early Eluting Pigment—HPLC-purified bilin was dried in vacuo and dissolved in anhydrous pyridine-D3 containing a trace of tetramethylsilane. The 400-MHz 1H NMR spectrum was recorded at room temperature. Because of the low concentration of bilin (approximately 0.25 mM) and concerns about sample stability at room temperature during the NMR spectroscopy, the sensitivity was maximized by using 10-μs (90°) pulses and 2.26-s data acquisition times. Three thousand scans, collected over a period of approximately 2 h, were signal-averaged. The 0.0-7.5-ppm region of the 1H NMR spectrum is shown in Fig. 4, and the properties of the relevant NMR signals are listed in Table II. Also tabulated are the 1H NMR signals of racemic 3(E)-phycocyanobilin dimethyl ester (Gossauer and Hirsch, 1974) and the phycocyanobilin undecapeptide from oat phytochrome (Lagaris and Rapoport, 1980) in pyridine-D3 and racemic 3(E)-phytochromobilin dimethyl ester in CDCl3 (Weller and Gossauer, 1980). Most of the proton signals correspond very closely to those of equivalent protons of the other pigments. It was shown previously that the chemical shifts of several biliverdin IXα proton signals are significantly influenced by the solvent (Beale and Cornejo, 1991b). This is particularly true for the downfield signals assigned to the 18-vinyl protons and the 10- and 15-methylene protons. This solvent effect accounts for the differences between the chemical shifts for these protons measured in pyridine-D3 and the values reported for 3(E)-phytochromobilin dimethyl ester recorded in CDCl3 (Weller and Gossauer, 1980).

All of the 1H NMR signals assigned to protons on the free bilin compared well with those that also occur on the peptide-bound phytochrome chromophore (Lagaris and Rapoport, 1980), except for the proton at the 2-position and those attributed to the 3-ethylidine group. These differences are consistent with the replacement of the ethylidine group of the free pigment by a thioethyl moiety in the phytochromobilin undecapeptide. Analysis of the signals assigned to the 18-vinyl group was complicated by the closeness of the chemical shifts of two of the protons, leading to a second order spectrum with distorted peak heights and anomalous shifts in peak...
Reverse-phase HPLC was on a 4.6-mm-diameter × 25-cm-long column of octadecysilane-coated 5-µm-diameter spherical silica particles (Altex). The elution solvent was ethanol/acetonewater/acetic acid (48:34:17:1, v/v) flowing at 4.0 ml/min. Visible absorption spectra of the HPLC-purified pigments were recorded in methanol/36% (w/v) aqueous HCl (49:1, v/v).

Characterization of bilins by HPLC elution times and visible absorption maxima

| Pigment Source                  | HPLC elution time | Absorption maxima |
|--------------------------------|-------------------|-------------------|
| Biliverdin IXα                  | 12.4* min         | 377,696* nm       |
| 3(Z)-Phyocyanobilin             | 17.1* min         | 369,686* nm       |
| 3(Z)-Phycocyanobilin            | 17.1* min         | 369,686* nm       |
| 3(Z)-Phycoerythrobilin          | 21.0* min         | 327,591* nm       |
| 3(Z)-Phytochromobilin           | 21.0* min         | 327,591* nm       |
| 3(E)-Phytochromobilin           | 23.2 min          | 386,700 nm        |
| 3(E)-Phytochromein              |                  |                   |
| 15,16-Dihydroxybiliverdin IXα   | 28.2* min         | 335,560* nm       |
| 3(E)-Phycocyanobilin            | 33.8* min         | 375,093* nm       |
| 3(E)-Phycoerythrobilin          | 33.8* min         | 375,093* nm       |
| 3(E)-Phytochromobilin           | 41.0* min         | 329,592* nm       |

*Beale and Cornejo (1991b).
*McDonagh (1979).
*Weller and Gossauer (1980) (data are for the dimethyl ester).
*Beale and Cornejo (1991c).
*Cole et al. (1967).
*Killilea et al. (1980).

Fig. 3. Absorption and CD spectra of 3(E)-phytochromobilin and 3(E)-phycoerythrobilin. 3(E)-Phytochromobilin was obtained by methanolysis of solvent-extracted P. cruentum cells. The pigment was purified by solvent partitioning, DEAE-Sepharose chromatography, and reverse-phase HPLC as described under “Results.” 3(E)-Phycocyanobilin was obtained by methanolysis of purified Synechococcus sp. PCC 7002 C-phycocyanin. Absorption (a) and CD (b) spectra were recorded in methanol/36% (w/v) aqueous HCl, 48:2 (v/v). Solid curves are the spectra of phytochromobilin, and dashed curves are the spectra of phycoerythrobilin.

The occurrence of small signals close to those assigned to the 5- and 15-methylene protons (Figs. 4 and 5) suggests that other bilins were generated while the pigment was standing at room temperature in pyridine during the NMR data acquisition period. After the 1H NMR spectrum was recorded, the solvent was evaporated in vacuo and the residue was analyzed for degradation by reverse-phase HPLC. Some degradation products were detected; the total A370 of these products was approximately 5% of that of the major component.

CD Spectroscopy of Methanolysis-derived 3(E)-Phytochromobilin—The CD spectra of methanolysis-derived 3(E)-phytochromobilin and 3(E)-phycocyanobilin were recorded in 36% (w/v) HCl/methanol (2:48, v/v). In this solvent, the bilins are fully protonated and free of helical conformations that can complicate interpretation of the spectra. Under these conditions, CD signals only reflect information about chiral carbons. Each of these bilins contains only one asymmetric carbon, at the 2-position (Fig. 1). The CD spectra of the two bilins are strikingly similar (Fig. 3b). The small differences in the positions of the peak wavelengths are expected from the similar differences in the absorption maxima (Fig. 3a).

Since the 2-carbon of natural phycocyanobilin is known to have the R configuration (Brockmann and Knoblach, 1973), the methanolysis-derived phytochromobilin also appears to have a 2R configuration.
phytochromobilin is biologically active, phytochrome assembly experiments were performed using recombinant oat apop-phytochrome prepared from yeast. For comparative purposes, a parallel experiment was performed using authentic 3(E)-phycocyanobilin dimethyl ester in CDC13 (Weller and Gossauer, 1980), 3.17–3.25 ppm (m) for 3(2)-phycoerythrobilin in pyridine-D6 (Beale and Cornejo, 1991b), 3.21 ppm (adjusted for the different reference standards) for 3(E)-phycoerythrobilin in pyridine-D6 (Crevis and Katz, 1969), and 3.24 ppm (q, J = 7) for 3(E)-phycoerythrobilin dimethyl ester in CDC13 (Gossauer and Weller, 1978).

The second order ABX spectrum of these interacting proton signals was analyzed with the aid of the Bruker PANIC NMR simulation and iteration program (see Fig. 5).

In conclusion, the early-eluting pigment obtained from P. cruentum was identified as 2(R),3(E)-phytochromobilin by comparative spectrophotometry, CD spectroscopy, and 1H NMR spectroscopy.

Holophytochrome Assembly Using P. cruentum-derived Phytochromobilin—To determine whether the 2(R),3(E)-phytochromobilin is biologically active, phytochrome assembly experiments were performed using recombinant oat apop-phytochrome prepared from yeast. For comparative purposes, a parallel experiment was performed using authentic 3(E)-phycocyanobilin, derived by methanolation of C-phycocyanin from Synechococcus sp. PCC 7002 cells. Covalent attachment of bilins to apophytochrome was first determined using a zinc blot assay. In this assay, bilin-linked polypeptides are visual-
Fig. 5. Analysis of the 5.60–6.90 ppm region of the 3(E)-phytochromobilin $^1$H NMR spectrum. The upper trace was produced by the Bruker PANIC NMR simulation and iteration program, using chemical shift values 5.548 ppm (X), 6.793 ppm (B), and 6.812 ppm (A), and coupling constants $J_{AB} = 17.541$ Hz, $J_{AX} = 10.574$ ppm, $J_{AX} = 3.668$ ppm. The lower trace is a portion of the spectrum of the pigment obtained from P. cruentum. The signals assigned to the 18-vinyl protons match the synthetic spectrum, the 5- and 15-methylene singlets are at 5.91 and 6.21 ppm, respectively, and the 3'-ethylinde quartet of doublets is at 6.35 ppm.

Table III

Ethylidine $^1$H NMR chemical shift values of 3(Z)- and 3(E)-ethylidine bilins

| Compound                  | Solvent       | Chemical shift ppm | Ref.          |
|---------------------------|---------------|--------------------|---------------|
| 3(E)-Phytochromobilin     | Pyridine-D$_6$ | 1.72, 6.35         | Our data      |
| 3(E)-Phytochromobilin     | CDCl$_3$      | 1.81, 6.38         | —             |
| 3(E)-Phycocyanobilin      | Pyridine-D$_6$| 1.64, 6.23         | —             |
| 3(E)-Phycocyanobilin      | Pyridine-D$_6$| 1.65, 6.24         | —             |
| 3(E)-Phycocyanobilin      | TFA-D         | 1.90, 6.68         | —             |
| 3(E)-Phycocyanobilin      | Pyridine-D$_6$| 1.83, 6.23         | —             |
| 3(E)-Phycocyanobilin      | TFA-D         | 1.85              | —             |
| 3(E)-Phycocyanobilin      | CDCl$_3$      | 1.89, 6.40         | —             |
| 3(E)-Phycocyanobilin      | CDCl$_3$      | 1.91, 6.40         | —             |
| 3(Z)-Phytochromobilin     | CDCl$_3$      | 2.12, 5.86         | —             |
| 3(Z)-Phycocyanobilin      | CDCl$_3$      | 2.09, 5.80         | —             |
| 3(Z)-Phycocyanobilin      | CDCl$_3$      | 2.08, 5.84         | —             |
| 3(Z)-Phycocyanobilin      | Pyridine-D$_6$| 2.18, 5.77         | —             |

$^a$ Weller and Gossauer (1980).

$^b$ Chemical shifts, which were originally reported relative to that of hexamethyldisiloxane, have been adjusted by adding 0.06 ppm to the published values to facilitate comparison with the other tabulated values, which are relative to that of tetramethylsilane.

$^c$ Cole et al. (1967).

$^d$ Gossauer and Hirsch (1974).

$^e$ Crespi and Katz (1969).

$^f$ Chapman et al. (1987).

$^g$ Gossauer and Weller (1978).

$^h$ Beale and Cornejo (1991b).

FIG. 6. Zinc blot analysis of reconstituted phytochromes. Apophytochrome-containing extracts from yeast cultures expressing the plasmid pPhyA3 were incubated with 3(E)-phytochromobilin (lane 3), 3(E)-phycocyanobilin (lane 1), or no bilin as a control (lane 2), then resolved by 7.5% T SDS-PAGE (1.5-mm thickness), transblotted to a poly(vinylidene difluoride) membrane, and assayed for zinc-dependent fluorescence as described under "Experimental Procedures." Lane 1 contains partially purified oat phytochrome extracted from etiolated seedlings as described previously (Elich et al., 1989). Sample loads were 200 ng of phytochrome protein per lane. For photography, the blot was exposed for 75 s using a red cutoff filter as described previously (Wahleithner et al., 1991).

quantitative immunoblot analyses (not shown), reconstitution yields in excess of 90% were estimated for both bilins in this experiment. The difference maximum and minimum of the phytochromobilin adduct at 666 and 730 nm, respectively, are nearly identical with the values of 668 and 732 nm reported for native oat phytochrome, within the resolution limits of the instruments used (Lagarias et al., 1987). By contrast, the difference maximum and minimum of the phycocyanobilin adduct were both blue-shifted as has been reported previously (Elich and Lagarias, 1989; Wahleithner et al., 1991). The $\Delta A_{max}/\Delta A_{min}$ ratio of the phytochromobilin adduct was 1.09, which is also in good agreement with the value of 1.14 reported previously for native oat phytochrome (Lagarias et al., 1987). All of the above spectrophotometric measurements were obtained with the HP 8450A diode-array spectrophotometer, which has a fixed 2 nm resolution in the visible region. For greater wavelength accuracy, an Aviv 14DS spectrophotome-
phytochromobilin and phycoerythrobilin-12-monomethyl ester (Rudiger, 1967b). From these extinction coefficients (used without correction for differences in wavelengths and solvents) and the relative peak areas of the HPLC elution profile, the molar ratio of phytochromobilin:phytocyanobilin:phycoerythrobilin in the methanolysis solution was calculated to be 1:6:3.2:9. Phytochromobilin was 27% of the total. Under the extraction and methanolysis conditions described above, each gram (fresh weight) of P. cruentum cells yielded approximately 10-15 nmol of purified 2(R),3(E)-phytochromobilin.

Requirements for Obtaining Phytochromobilin—Since P. cruentum cells do not contain phytochromobilin or phytochromobilin-bearing phycobiliproteins, this bilin must have arisen by chemical modification of phycobiliprotein chromophores. To determine which specific step in the cell extraction and methanolysis procedures were required for the production of phytochromobilin, these steps were varied systematically. During the course of the studies discussed above, it was found that P. cruentum cells, unlike C. caldarium, do not require dimethyl sulfoxide for the initial cell permeabilization; acetone alone is sufficient. Cells were extracted several times with acetone, until the extraction supernatants were colorless, and then methanolized. These cells yielded pigment mixtures in which phytochromobilin was a major component (data not shown). Therefore, exposure of the cells to dimethyl sulfoxide is not required to obtain phytochromobilin. It was not possible to directly examine the dependence on acetone for obtaining phytochromobilin from P. cruentum cells, because acetone is required either for permeabilizing the cells or for sedimenting cells that are permeabilized with dimethyl sulfoxide. When cells that had been permeabilized with acetone were washed extensively with methanol to remove residual acetone before methanolysis, they aggregated into clumps that were resistant to methanolysis.

Cells of the filamentous cyanobacterium, Calothrix sp. PCC 7601, unlike P. crucentum and C. caldarium, can be permeabilized with methanol. After extraction of the cells several times with methanol, until the extraction supernatants were colorless, methanolysis yielded phycoerythrobilin and phycocyanobilin, but no phytochromobilin. To test the effect of acetone, methanol-extracted cells were washed with acetone, and the acetone was then removed by centrifugation. In this case, methanolysis yielded all three bilins (data not shown). All three bilins were also produced when methanol-extracted cells were methanolized in the presence of controlled concentrations of acetone. The yield of phytochromobilin was approximately equal when the methanolysis solution contained acetone within the range of 0.5-10% (v/v) (data not shown). To test whether the effect of acetone was due to the presence of a contaminant in commercial reagent grade acetone, a methanolysis was done with methanol containing freshly redistilled acetone. This methanolysis yielded about the same amount of phytochromobilin as those containing commercial acetone (data not shown).

To determine whether the effect of acetone is exerted before or after the bilins are released from the phycobiliproteins by methanolysis, Calothrix cells were extracted with methanol and then divided into two portions for methanolysis. One portion was methanolized for 12 h at 40 °C in methanol/HgCl₂ and other in methanol/HgCl₂ containing 2% (v/v) acetone. After separation of the solvent, the cell residues from both methanolysis reactions were washed extensively with methanol. The cells from the acetone-free first methanolysis were methanolized for 16 h at 40 °C with methanol/HgCl₂ containing 2% (v/v) acetone. After separation of the solvent, the cell residues from both methanolysis reactions were washed extensively with methanol. The cells from the acetone-free first methanolysis were methanolized for 16 h at 40 °C with methanol/HgCl₂ containing 2% (v/v) acetone, and the cells from the acetone-containing first methanolysis were methanolized for 16 h with methanol/HgCl₂ in the absence of acetone. Finally, portions of the methanol solution from the first acetone-free methanolysis were held at 40 °C for 16 h in the presence and absence of 2% (v/v) acetone. Phytochromobilin was detected in the products of both the first and second methanolysis incubations of the cells that were first methanolized in the presence of acetone (data not shown). Phytochromobilin was detected only in the products of the second methanolysis (in the presence of acetone) of the cells that were first methanolized in the absence of acetone. Addition of 2% (v/v) acetone to the methanolysis products of cells that were methanolized in the absence of acetone did not yield phytochromobilin upon
Phytochromobilin Structure and Activity

Phytochromobilin is derived from phycoerythrin, as shown by x-ray crystallography and chemical degradation. The configuration of the phytochromobilin chromophore is 2R,3R,3'S, indicating that the absolute configuration is either 2R,3R or 2S,3S. The existence of a strong CD signal for the 2-carbon of the bilin was not racemized during the oxidation of protein-bound phycoerythrobilin and methanolysis. These results support the hypothesis that phytochromobilin is formed by conversion of a phycobilin chromophore to phytochromobilin in addition to the expected phycobilins, phycoerythrobilin, and phycocyanobilin. Comparative spectroscopic measurements have confirmed that the released phytochromobilin has the 3E configuration of the ethylidene double bond and an R stereochemistry at the 2-carbon. Evidence for the correct assembly of 2(R),3(E)-phytochromobilin with recombinant oat apophytochrome to form native holophytochrome includes formation of a covalent bond between bilin and apoprotein and the similarity of the difference spectrum of the reconstituted phytochrome with that of native oat phytochrome. Previous reconstitution experiments using apophytochrome and phycocyanobilin have also yielded a covalently bonded adduct that underwent photoreversible spectral changes, but the difference spectrum differed from that of native phytochrome, reflecting the substitution of phycocyanobilin for the natural phytochrome chromophore precursor. The generation of the native photoreversible difference spectrum in the reconstitutions with 2(R),3(E)-phytochromobilin strongly suggests that this bilin is the natural in vivo phytochrome chromophore precursor.

DISCUSSION

These studies have established that methanolysis of solvent-extracted P. cruentum and Calothrix cells releases phytochromobilin in addition to the expected phycobilins, phycocyanobilin, and phycoerythrobilin. Comparative spectroscopic measurements have confirmed that the released phytochromobilin has the 3E configuration of the ethylidene double bond and an R stereochemistry at the 2-carbon. Evidence for the correct assembly of 2(R),3(E)-phytochromobilin with recombinant oat apophytochrome to form native holophytochrome includes formation of a covalent bond between bilin and apoprotein and the similarity of the difference spectrum of the reconstituted phytochrome with that of native oat phytochrome. Previous reconstitution experiments using apophytochrome and phycocyanobilin have also yielded a covalently bonded adduct that underwent photoreversible spectral changes, but the difference spectrum differed from that of native phytochrome, reflecting the substitution of phycocyanobilin for the natural phytochrome chromophore precursor (Elich et al., 1989; Elich and Lagarias, 1989; Lagarias and Lagarias, 1989; Wahleitner et al., 1991; Deforce et al., 1991). The generation of the native photoreversible difference spectrum in the reconstitutions with 2(R),3(E)-phytochromobilin strongly suggests that this bilin is the natural in vivo phytochrome chromophore precursor.

The spontaneous assembly of phytochromobilin and phycocyanobilin with apophytochrome to form spectrally active covalent adducts is in distinct contrast to the inability of phycocyanobilin to correctly assemble with apophytochrome. With apophycocyanin, although covalent adducts were formed, they were not equivalent to native holophycoerythrin but differed with respect to spectral properties, peptide chromatographic behavior, and structure of the bound chromophores (Arciero et al., 1989a, 1989b). These results suggest that phycobiliprotein assembly requires additional enzymes and/or cofactors. Phytochromobilin is probably not naturally present in P. cruentum or Calothrix cells. Its occurrence is unlikely to have been missed in earlier detailed studies of the phycobiliprotein chromophore content of these organisms, especially if it is present at the 27% mole fractional content that was estimated for the P. cruentum methanolysis products. In addition, neither of these organisms has been reported to contain phytochrome or have phytochrome responses. Moreover, the pigment was obtained only when cells were exposed to acetone before or during methanolysis. Therefore, it seems probable that phytochromobilin is formed by conversion of a phycobiliprotein chromophore during methanolysis. Our studies point to phycoerythrin as the source of this pigment. First, phytochromobilin was obtained only from phycoerythrin-containing cells. Second, the transformation required for conversion of the phycoerythrin chromophore to phytochromobilin involves the oxidation of a methylene bridge (Fig. 1), a reaction that is known to occur readily in bilins. For example, bilirubin (10,11-dihydrobiliverdin) is readily oxidized to biliverdin, both chemically (McDonagh, 1979; McDonagh and Palma, 1980) and enzymatically (Murao and Tanaka, 1982). By contrast, the required chemical transformation necessary for converting phycocyanin chromophore to phytochromobilin entails the more difficult oxidation of an ethyl group to a vinyl group.

Together with the observation that acetone treatment prior to methanolysis is required for phytochromobilin production, these results support the hypothesis that phytochromobilin is derived from the bilin prosthetic group(s) of phycoerythrin. In P. cruentum, phycoerythrin contains a third phycobilin chromophore, phycourobilin, in addition to phycocyanobilin and phycoerythrobilin (Glazer and Hixson, 1977), which raises the possibility that phytochromobilin is derived from the protein-linked phycourobilin chromophore. This possibility is excluded because Calothrix phycoerythrin contains no phycourobilin (Beguin et al., 1985; Glazer, 1988) but yields the 2-, 3-, and 3'-protons of phytochromobilin undecapeptide are also very similar to those of the C-phycocyanin β1 chromophore (Lagarias et al., 1979). The latter has recently been shown by x-ray crystallography to have the 2R,3R,3'S configuration (Schirmer et al., 1987; Duerring et al., 1991), confirming earlier predictions based on chemical degradation results (Klein and Rüdiger, 1978). It was also predicted from chemical degradation that the configuration of the phytochrome chromophore is 2R,3R,3'S, but the 2S,3S,3'S configuration could not be excluded (Klein et al., 1977). From our direct determination, by comparative CD spectroscopy, that the configuration at C-2 is also R and that the reconstituted phytochrome chromophore is 2R,3R,3'S. In view of the high reconstitution efficiency and the spectral indistinguishability of the reconstituted adduct from natural phytochrome, it is highly unlikely that their chromophores differ.

Further incubation for 16 h at methanolysis temperature.

To test whether phytochromobilin is derived from phycoerythrin, similar experiments were performed with Synechocystis sp. PCC 6803, a unicellular cyanobacterium whose cells contain only phycocyanobilin-bearing phycobiliproteins. Synechocystis cells that were extracted and methanolized identically with the conditions that produced phytochromobilin from Calothrix cells yielded only phycocyanobilin; neither phycoerythrobilin nor phytochromobilin was detected as methanolysis products. Similar experiments with C. caldarium, which also lacks phycoerythrin, produced no phytochromobilin recovery, regardless of the presence of acetone in the extraction and methanolysis solutions (data not shown).

From these results, it appears that acetone acts on protein-bound phycoerythrobilin to facilitate oxidation at the 15,16-position to form protein-bound phytochromobilin, which is subsequently cleaved from the protein by methanol to yield free phytochromobilin. Formation of phytochromobilin is also not specifically dependent on HgCl₂, but the presence of HgCl₂ in the methanolysis medium increases the yield of all free bilins (data not shown).

Previous predictions of the absolute configuration of the phytochrome chromophore have been based on analogy with those of phycobilins. The existence of a strong CD signal for the 2-carbon of the bilin was not racemized during the oxidation of protein-bound phycoerythrobilin and methanolysis. The CD spectrum shows that the configuration at the 2-position of 3(E)-phytochromobilin is the same as that of 3(E)-phycocyanobilin from methanolytically cleaved C-phycocyanin. The absolute configurations at C-2 of phycoerythrobilin-generated 3(E)-phycocyanobilin and 3(E)-phycoerythrobilin have previously been shown to be R (Broekmann and Knobloch, 1973; Gossauer and Weller, 1978). The magnitude of coupling constants between the C-2 and C-3 protons in the 1H NMR spectrum of oat phytochromobilin undecapeptide showed that the C-2 and C-3 substituents are trans, indicating that the absolute configuration is either 2R,3R or 2S,3S (Lagarias and Rapport, 1980). The coupling constants among the 2-, 3-, and 3'-protons of phytochromobilin undecapeptide are also very similar to those of the C-phycocyanin β1 chromophore (Lagarias et al., 1979). The latter has recently been shown by x-ray crystallography to have the 2R,3R,3'S configuration (Schirmer et al., 1987; Duerring et al., 1991), confirming earlier predictions based on chemical degradation results (Klein and Rüdiger, 1978). It was also predicted from chemical degradation that the configuration of the phytochrome chromophore is 2R,3R,3'S, but the 2S,3S,3'S configuration could not be excluded (Klein et al., 1977). From our direct determination, by comparative CD spectroscopy, that the configuration at C-2 of the 3(E)-phytochromobilin used for reconstitution is R, together with the foregoing conclusions, it follows that the configuration at C-3 is also R and that the reconstituted phytochrome chromophore is 2R,3R,3'S. In view of the high reconstitution efficiency and the spectral indistinguishability of the reconstituted adduct from natural phytochrome, it is highly unlikely that their chromophores differ.
phytochromobilin upon methanolysis. It is therefore concluded that phytochromobilin is derived from the phycoerythrobilin chromophore of phycoerythrin.

At this time, neither the identity of the proposed oxidant nor the role of acetone in facilitating the oxidation is known. It must be stressed that methanolysis was performed with whole cells. Although soluble materials were extracted with organic solvents before methanolysis, materials that could serve as the oxidant may have remained in the cells. The role of HgCl₂ in increasing the yield of bilins may be to react with the liberated bilins (Manitto and Monti, 1979). Another role for HgCl₂ could be to promote denaturation and unfolding of the phycobiliproteins, thereby increasing the accessibility of the bilin-cysteine thioether bonds to attack by methanol. A third possible role for HgCl₂ may be to inactivate chemical and enzymatic bilin degradation during methanolysis.

The availability of relatively large quantities of phytochromobilin now enables us to address a number of important questions related to phytochrome chromophore biosynthesis and holophytochrome assembly. For example, HPLC studies have already revealed that (E)-phytochromobilin is enzymatically produced from biliverdin IXα by isolated cucumber whole cells. Although soluble materials were extracted with organic solvents before methanolysis, materials that could condense with the liberated bilins (Manitto and Monti, 1979). Another role for HgCl₂ could be to promote denaturation and unfolding of the phycobiliproteins, thereby increasing the accessibility of the bilin-cysteine thioether bonds to attack by methanol. A third possible role for HgCl₂ may be to inactivate chemical and enzymatic bilin degradation during methanolysis.

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