Purification and Biochemical Properties of Phytochromobilin Synthase from Etiolated Oat Seedlings

Michael T. McDowell2 and J. Clark Lagarias*
Section of Molecular and Cellular Biology, University of California, One Shields Avenue, Davis, California 95616

Plant phytochromes are dependent on the covalent attachment of the linear tetrapyrrole chromophore phytochromobilin (PΦB) for photoactivity. In planta, biliverdin IXα (BV) is reduced by the plastid-localized, ferredoxin (Fd)-dependent enzyme PΦB synthase to yield 3Z-PΦB. Here, we describe the >50,000-fold purification of PΦB synthase from etioplasts from dark-grown oat (Avena sativa L. cv Garry) seedlings using traditional column chromatography and preparative electrophoresis. Thus, PΦB synthase is a very low abundance enzyme with a robust turnover rate. We estimate the turnover rate to be >100 s⁻¹, which is similar to that of mammalian NAD(P)H-dependent BV reductase. Oat PΦB synthase is a monomer with a subunit mass of 29 kD. However, two distinct charged forms of the enzymes were identified by native isoelectric focusing. The ability of PΦB synthase to reduce BV is dependent on reduced 2Fe-2S Fds. A Km for spinach (Spinacea oleracea) Fd was determined to be 3 to 4 μM. PΦB synthase has a high affinity for its bilin substrate, with a sub-micromolar Km for BV.

As photosynthetic organisms, plants continually monitor and respond to changes in their light environment. Therefore, plants have evolved numerous photoperception and signaling systems to modulate growth and development in response to light (Neff et al., 2000). The family of phytochromes in higher plants, cryptophytes, and cyanobacteria are the most extensively studied of these photoreceptors (Quail, 1991; Pepper, 1998; Davis et al., 1999b). Holophytochromes are homodimers consisting of 124-kD protein subunits with a covalently attached linear tetrapyrrole chromophore phytochromobilin (PΦB). The chromophore molecule enables the holoprotein to exist in two spectrally distinct light absorbing forms—Pr, the red light-absorbing form, and Pfr, the far-red light-absorbing form—that are vital to the mechanism of action of phytochromes.

Previous experiments have established that the biosynthesis of linear tetrapyrrole precursors of the phytochrome chromophore occurs entirely within the plastid organelle (Terry and Lagarias, 1991; Terry et al., 1993). PΦB then is released into the cytosol where the autocatalytic assembly with nascent apophytochrome in the plant cell cytoplasm (Terry et al., 1993).

The first committed step in the synthesis of PΦB (see Fig. 1) is the ring opening of heme to yield biliverdin IXα (BV)—a reaction catalyzed by a ferredoxin (Fd)-dependent heme oxygenase. Fd-dependent heme oxygenases were first identified in red algae and cyanobacteria (Rhie and Beale, 1992; Rhie and Beale, 1995; Cornejo and Beale, 1997; Cornejo et al., 1998). The HY1 gene encodes an orthologous enzyme in Arabidopsis, one of four to five heme oxygenase genes in this species (Davis et al., 1999a; Muramoto et al., 1999).

In the unicellular red alga Cyanidium caldarium, BV is the substrate for at least two Fd-dependent reduction processes that are required for the biosynthesis of the phycobiliprotein chromophore precursors, phycoerythrobilin and phycoerythrobilin (Beale and Cornejo, 1991a, 1991b, 1991c). In contrast, BV is directly reduced to 3Z-PΦB in plants by the Fd-dependent enzyme PΦB synthase, whose gene HY2 was recently cloned from Arabidopsis (Kohchi et al., 2001). 3Z-PΦB has been shown to be a functional precursor of the phytochrome chromophore; however, its isomerization to 3E-PΦB is thought to occur prior to covalent attachment to apophytochrome in the plant cell cytoplasm (Terry et al., 1993).

Although the demonstration that the HY2 gene of Arabidopsis encodes a functional PΦB synthase is a significant advance (Kohchi et al., 2001), it is necessary to study PΦB synthase isolated from plants to ensure that the biochemical properties of the recombinant enzyme accurately reflect those of the enzyme isolated from natural sources. This paper presents the first reported purification and biochemical characterization of a PΦB synthase from plants. Oat (Avena

---

1 This work was supported by the U.S. Department of Agriculture National Research Initiative Competitive Grants Program (grant no. AMD-9801768 to J.C.L.).
2 Present address: Amersham Pharmacia Biotech, 654 Minnesota Street, San Francisco, CA 94107-0387.
* Corresponding author, e-mail jclagarias@ucdavis.edu; fax 530–752–3085.
na sativa L. cv Garry) seedlings were chosen for these studies, owing to the availability of a large quantity of tissue and the high level of \( \Phi \)-9021B synthase activity in isolated plastid preparations from this species (Terry and Lagarias, 1991). Because earlier studies showed that \( \Phi \)-9021B synthase is a plastid-localized enzyme, etioplast preparations were used to enrich the activity of the enzyme in the crude homogenate. 

Relying on two assay systems, a coupled assay employing apophytochrome for detection of holophytochrome activity (Terry and Lagarias, 1991) and a direct HPLC-based assay for the detection of \( \Phi \)-9021B isomers (Wu et al., 1997), we describe a >50,000-fold purification and initial biochemical characterization of this low abundance enzyme using conventional methodologies.

**RESULTS**

**Purification of \( \Phi \)-9021B Synthase**

Our lab previously documented that \( \Phi \)-9021B synthase activity is localized to the plastid organelle of higher plants and green algae (Terry et al., 1993, 1995). For this reason, etioplasts were used as starting material for the purification of \( \Phi \)-9021B synthase. Etioplasts were resuspended in iso-osmotic buffer and osmotically lysed by dilution with 10 volumes of buffer lacking osmoticum. Initial experiments using TES [N-tris (hydroxymethyl)-2-aminoethanesulfonic acid]/HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid] lysis buffers indicated that \( \Phi \)-9021B synthase activity remained membrane associated, with detergent treatments and high salts washes not yielding soluble, active protein. Substitution of the TES/HEPES with potassium phosphate buffer solubilized 30% to 50% of the total \( \Phi \)-9021B synthase activity found in the crude intact etioplasts (Table I). The phosphate buffer appears to act as a metal ion chelator because omission of \( \text{Mg}^{2+} \) ion and addition of EGTA in the lysis buffer further enhanced the recovery of solubilized \( \Phi \)-9021B synthase activity.

A 50% to 70% saturated ammonium sulfate fractionation protocol was devised to optimize recovery/purification of \( \Phi \)-9021B synthase and concentration of the protein extract (Table I). The use of anion-exchange chromatography was next explored using a HiTrapQ cartridge to which the enzyme bound strongly at low ionic strength. It is unfortunate that the recovery of \( \Phi \)-9021B synthase activity with this methodology was low (i.e. approximately 30%). Apparently due to irreversible adsorption to the HiTrapQ cartridge and/or to denaturation of the enzyme at low ionic strength, we found that raising the ionic strength to 60 mM KCl in both application and pre-equilibration buffers led to nearly quantitative recovery of \( \Phi \)-9021B synthase activity in the column flow-through. This result was unexpected because this concentration of KCl was well below that determined to elute the enzyme activity bound to the column in the original experiments. At this pH and ionic strength, \( \Phi \)-9021B synthase bound strongly to a HiTrapBlue (Cibacron Blue) dye ligand cartridge but not to a cation-exchange HiTrapSP cartridge. Taking advantage of these observations, the 50% to 70% ASP sample was applied to a tandem series of HiTrapQ, HiTrapSP, and HiTrapBlue cartridges in a pH 7.5 buffer containing 60 mM KCl. In this way, \( \Phi \)-9021B synthase could be

---

**Figure 1.** Biosynthesis of \( \Phi \)-9021B from heme. The linear tetrapyrrole precursor of the plant phytochrome chromophore is synthesized from heme via the subsequent reactions of hemeoxygenase and \( \Phi \)-9021B synthase. The obtained 3Z-\( \Phi \)-9021B isomer is readily isomerized to its 3E-form, both of which are functional precursors of the phytochrome chromophore.
recovered bound to the HiTrapBlue cartridge, whereas many impurities had adsorbed to both ion-exchange cartridges. PΦB synthase activity was then eluted from the HiTrapBlue cartridge with a 2 mM KCl gradient as shown in Figure 2. The HiTrapBlue eluted fractions containing PΦB synthase (THC) were then pooled and further fractionated with ceramic hydroxyapatite Econo-Pac CHT-II (CHT-II) and gel filtration chromatography (GFC). Elution profiles for these columns are shown in Figure 2. Protein molecular mass standards were used to estimate the molecular mass of PΦB synthase to be 25 kD (Fig. 3).

As shown in Table I, PΦB synthase could be purified more than 8,000-fold with an overall yield of 40% using a combination of organelle isolation, ammonium sulfate fractionation, THC, CHT-II, and GFC. Because SDS-PAGE revealed multiple protein components (Fig. 4), further purification of PΦB synthase was needed. Native isoelectric focusing (IEF) was examined because of its high resolution and non-denaturing conditions that permitted assay of PΦB synthase. The pooled PΦB synthase fractions from GFC were focused on an IEF PHAST gel, pH 6.5 to 3.0 (Amersham Pharmacia Biotech, Piscataway, NJ). A portion of the gel was sliced into 2-mm sections and assayed for PΦB synthase activity with the coupled assay, whereas the remainder of the gel was silver stained (Fig. 5). PΦB synthase activity was associated with two protein bands with pIs of 5.7/5.6 and 5.3/5.2, with the higher pI form predominating. No activity was detected in the protein that migrated to the high pH end of the gel. Both pI 5.7/5.6 and pI 5.3/5.2 species were excised from the gel, resolved by SDS-PAGE, and silver-stained. In both lanes, a major protein of molecular mass 29 kD was observed (data not shown).

Based on protein and enzymatic activity measurements, we estimate an overall purification of more than 50,000-fold. Despite valiant attempts (i.e. excision of the bands from the IEF gel, SDS-PAGE, in gel trypsin digestion of the 29-kD band, peptide purification by HPLC, and protein microsequence analysis), we have so far been unable to obtain protein sequence that has enabled us to clone the gene encoding PΦB synthase. The recent cloning of HY2 should facilitate the cloning of the gene encoding PΦB synthase from oats (Kohchi et al., 2001).

**Table 1. Purification of PΦB synthase from etiolated oat seedlings**

Starting with 16 kg of 1-week-old etiolated oat seedlings, PΦB synthase was purified 8,000-fold with a 45% yield. The arbitrary units (AU) are obtained by dividing the HPLC peak area for 3Z-PΦB by its molar absorption coefficient and the time of the assay in minutes.

<table>
<thead>
<tr>
<th>Purification Fraction</th>
<th>Intact</th>
<th>Soluble</th>
<th>ASP</th>
<th>THC</th>
<th>CHT-II</th>
<th>GFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total volume (mL)</td>
<td>168</td>
<td>332</td>
<td>42</td>
<td>2.75</td>
<td>6.50</td>
<td>0.30</td>
</tr>
<tr>
<td>Total protein (mg)</td>
<td>2,055</td>
<td>743</td>
<td>216</td>
<td>3.25</td>
<td>1.05</td>
<td>0.113</td>
</tr>
<tr>
<td>S.A. (AU mg⁻¹)</td>
<td>0.064</td>
<td>0.178</td>
<td>0.638</td>
<td>13.3</td>
<td>54.8</td>
<td>526</td>
</tr>
<tr>
<td>Total activity (AU)</td>
<td>132</td>
<td>132</td>
<td>138</td>
<td>43.2</td>
<td>57.7</td>
<td>59.2</td>
</tr>
<tr>
<td>Yield (%)</td>
<td>100</td>
<td>100</td>
<td>105</td>
<td>32.8</td>
<td>44</td>
<td>45</td>
</tr>
<tr>
<td>Fold purification</td>
<td>1</td>
<td>2.8</td>
<td>10.0</td>
<td>208</td>
<td>855</td>
<td>8,207</td>
</tr>
<tr>
<td>Step yield (%)</td>
<td>–</td>
<td>–</td>
<td>104</td>
<td>31.3</td>
<td>134</td>
<td>103</td>
</tr>
<tr>
<td>Step fold purification</td>
<td>–</td>
<td>–</td>
<td>3.6</td>
<td>20.8</td>
<td>4.1</td>
<td>9.6</td>
</tr>
</tbody>
</table>

**Biochemical Characterization of PΦB Synthase**

Lacking a continuous assay for PΦB synthase assay, we relied on the HPLC analysis to study the BV substrate and Fd cofactor dependence. All kinetic measurements were performed using PΦB synthase preparations obtained from pooled GFC fractions. To establish that initial rate conditions were satisfied, aliquots were removed from the assay mixture at various timepoints, bilins were isolated by C18 Sep-Pak solid-phase extraction and then analyzed by HPLC using the conditions described previously (Wu et al., 1997) and in “Materials and Methods.” These studies showed first order production of 3Z-PΦB within the first half hour of assay, enabling us to use a fixed time analysis for enzyme activity determination (data not shown). Only 3Z-PΦB product was detected and the activity was expressed as the integrated area under the 3Z-PΦB peak detected at 380 nm (Wu et al., 1997). Based on standardization of HPLC traces (data not shown), we estimate a minimum turnover rate of oat PΦB synthase to be 106 min⁻¹.

To study the Fd dependence of PΦB synthase, commercially available Fd preparations from spinach (*Spinacea oleracea*), *Porphyra umbilicalis*, *Spirulina* sp., and *Clostridium pasteurianum* were compared. All Fd preparations, except that from *C. pasteurianum*, the only 4Fe-4S Fd, could support BV reduction to 3Z-PΦB. The kinetic data for spinach Fd was plotted using both the Lineweaver-Burk (1/v versus 1/[Fd]) and Eadie-Scatchard methods (v/[Fd] versus v) to obtain an estimate of Kₘ and V_max. The Lineweaver-Burk plot is shown in Figure 6. Based on the average of these two analyses, the apparent Kₘ of PΦB synthase for spinach Fd was 3.5 μM. The V_max for PΦB synthase was estimated to be 3.3 AU min⁻¹. When the kinetic data was plotted using the Eadie-Scatchard method, the data appears to deviate from linearity at the lower Fd concentrations. This result may be due to the complex assay mixture required to assay PΦB synthase, and in particular, the differential affinity of the spinach Fd for Fd:NAD⁺ oxi-
doreductase (FNR) and oat PΦB synthase. The apparent \( K_m \) for \( P. \) umbilicalis Fd was 1.2 \( \mu \)M, which was much lower than that of spinach Fd, whereas the apparent \( V_{\text{max}} \), at 2.0 AU min\(^{-1}\), was slightly lower than that for spinach. For \( Spirulina \) sp. Fd, the observed values were slightly higher than those for \( P. \) umbilicalis Fd but lower than those for spinach Fd. The apparent \( K_m \) for \( Spirulina \) sp. Fd was 1.8 \( \mu \)M and the apparent \( V_{\text{max}} \) was 3.91 AU min\(^{-1}\), much closer to that for spinach (plots not shown).

Experiments also were undertaken to estimate the affinity of PΦB synthase for BV. These measurements indicate that the concentrations tested (>3.3 \( \mu \)M), the limit of practical detection by our analytical HPLC system, were well in excess of the \( K_m \) for biliverdin (data not shown). No difference in product formation was observed at all BV concentrations tested; therefore, the \( K_m \) for BV appears to be sub-micromolar.

DISCUSSION

Using a combination of precipitation, adsorption chromatography, and electrophoretic methods, PΦB synthase from oat etioplasts has been purified >50,000-fold to near homogeneity. This has enabled us to examine the biochemical properties of this enzyme from a natural source for the first time. Because the gene encoding PΦB synthase HY2 has been recently cloned from the dicot species Arabidopsis (Kohchi et al., 2001), the isolation of the homologous genes in oat will facilitate direct comparisons between the natural and recombinant PΦB synthases. A detailed understanding of the biochemical properties of this enzyme should provide insight into the role PΦB synthase plays in the regulation of light sensitivity in situ.
Our results indicate that PΦB synthase is a low abundance enzyme in oats with a relatively high turnover rate. The low expression of PΦB synthase is underscored by the lack of cDNA clones for HY2 (or HY2-related) genes in the plant expressed sequence tag databases (Kohchi et al., 2001). The minimum turnover rate of oat PΦB synthase was estimated to be 106 min⁻¹, which is similar to that of rat NADPH-dependent BV reductase reported to be 102 min⁻¹ (Kutty and Maines, 1981). Both biliverdin reductases possess considerably higher turnover rates than those of the NADPH-dependent mammalian heme oxygenases, i.e. 2.4 min⁻¹ and 0.73 min⁻¹ for the respective rat and human enzymes (Yoshida and Kikuchi, 1979; Wilks et al., 1996), and that of the Fd-dependent heme oxygenase HO1 from Synechocystis sp. PCC6803, i.e. 0.009 min⁻¹ (Cornejo et al., 1998). From these estimates, heme oxygenase may be rate limiting for the formation of PΦB in plants. However, this will depend on the relative levels of heme oxygenase and PΦB synthase enzyme activities.

Based on GFC and SDS PAGE, oat PΦB synthase behaves as a monomer with a subunit molecular mass of 29 kD. This is similar to the predicted molecular mass of 33 kD for Arabidopsis PΦB synthase, which is encoded by the HY2 locus (Kohchi et al., 2001), and to the subunit molecular size of other recently described Fd-dependent bilin reductases (Wüthrich et al., 2000; Frankenberg et al., 2001). In the present study, however, we observed two species of PΦB synthase from etiolated oat seedlings with distinct pI. This suggests posttranslational processing (e.g. proteolysis, phosphorylation, etc.) of the oat enzyme, but does not rule out the possibility of multiple genes. Because oats cv Garry are a hexaploid species, which probably accounts for the presence of multiple phytochrome A genes (Hershey et al., 1985), it is reasonable that PΦB synthase is encoded by multiple genes in oats. This contrasts with Arabidopsis, whose genome possesses only a single PΦB synthase gene, HY2 (Kohchi et al., 2001).

Oat PΦB synthase has an apparent K_m for spinach Fd of 3 μM. Although the K_m for oat Fd does not appear to be much different, these measurements displayed complex kinetics, perhaps due to the use of dicot FNR to reduce monocot Fd. The weak affinity for Fd probably accounts for the inability of this enzyme to bind to spinach Fd-agarose affinity columns, a methodology that has been used successfully for the purification of Fd-dependent bilin reductases from the red alga C. caldarium (Beale and Cornejo, 1984, 1991a, 1991b). The present studies show that oat PΦB synthase also can utilize 2Fe-2S Fds from the algal species P. umbilicalis and Spirulina sp., whereas the 4Fe-4S Fd from Clostridium pasteurianum does not support BV reduction. However, we do not believe that the lower K_m values determined for P. umbilicalis

![Figure 4. SDS-PAGE of PΦB synthase purification. One microgram of total protein from each fraction of the PΦB synthase purification was loaded onto a 12.5% (w/v) polyacrylamide gel, electrophoresed, and stained with silver. STD, M_r standard; Lane 1, intact plastids; Lane 2, soluble fraction; Lane 3, 50% to 70% ammonium sulfate fraction; Lane 4, tandem HiTrap eluant; Lane 5, ceramic hydroxyapatite eluant; Lane 6, gel filtration chromatography.](image)

![Figure 5. IEF of PΦB synthase. A small amount of activity from the gel filtration step of the PΦB synthase purification was electrophoresed in triplicate on a Pharmacia pH 6.5 to 3.0 IEF PHAST gel using the PHAST system. Two lanes of the gel were sliced into 2-mm sections, removed from the solid support, and allowed to diffuse for 3 h at 4°C in 100 μM bovine serum albumin (BSA) and 100 mM potassium phosphate (pH 7.4). After diffusion, the slices were assayed for PΦB synthase activity using the coupled assay. The remainder of the gel was silver stained. ±, Indicates the presence or absence of PΦB synthase activity in each gel slice indicated by the marks on the left.](image)
and *Spirulina* sp. Fds are significant. A more exact determination of the $K_m$ (Fd) must await the development of a more direct method to reduce Fd as the use of FNR and and NADPH regenerating system complicates the assay. Although chemical reductants such as methyl viologen could be used, electrochemical methodologies will be pursued because of potential side reactions of the chemical reductants with bilin substrates and products.

Several key aspects of PΦB synthase activity await further characterization. Most notably, accurate determination of the bilin affinity will necessitate development of continuous, more sensitive assay methodologies. Our studies indicate that the $K_m$ for BV is sub-micromolar; however, the HPLC assay precludes accurate measurements in this range. Preliminary experiments indicate that BV forms a stable complex with recombinant HY2 in the absence of Fd (N. Frankenberg and J.C. Lagarias, unpublished data). Because the BV concentration in plant cells is not expected to reach the micromolar level, the high affinity for BV may be necessary for adequate synthesis of PΦB. It also is conceivable that PΦB synthase forms a complex with heme oxygenase in plant plastids, thereby channeling BV to PΦB synthase without its release. These questions will be the subject of future studies on the Fd-dependent bilin reductase family of enzymes.

**MATERIALS AND METHODS**

**Reagents**

Reagent grade or better chemicals, obtained from either Sigma Chemical Company (St. Louis) or Fisher Scientific (Pittsburgh) were used unless otherwise specified. HPLC grade solvents were obtained from Fisher Scientific. Trifluoroacetic acid and 4-methylmorpholine were distilled prior to use. All auxiliary enzymes and proteins were obtained from either Sigma or as gifts (as noted). Chromatographic resins were obtained from Bio-Rad (Hercules, CA), Sigma, Amershams Pharmacia Biotech, Phenomenex (Torrence, CA), or Whatman (Kent, UK). Sep-Pak cartridges were obtained from Waters Chromatography (Milford, MA). Oats (*Avena sativa* L. cv Garry) were obtained from Maine Potato Growers (Presque Island, ME). Fd was obtained commercially (Sigma), was purified from spinach (*Spinacea oleracea*; Buchanan and Arnon, 1971), or was received in purified form as a gift from Richard Malkin (University of California, Berkeley). BV was synthesized from bilirubin IXα as described previously (McDonagh and Palma, 1980). Recrystallized BV was used directly after synthesis or was further purified by reversed-phase HPLC. Purified BV was dissolved in Me2SO prior to use and quantitated by $A_{370}$ following dilution of an aliquot into 2% (v/v) HCl in methanol using a molar absorption coefficient of 66.2 mm$^{-1}$ cm$^{-1}$ (McDonagh and Palma, 1980).

**PΦB Synthase Purification**

Oat seeds (200 g) were imbibed in a solution of CaCl$_2$ (0.6 g L$^{-1}$) in distilled water at 4°C for 24 h. The imbibing solution was decanted and seeds were left at 4°C for an additional 24 h. Seeds were planted on cheesecloth-covered Vermiculite, covered with foil, and placed in a dark growth chamber at 24°C. Eight-day-old etiolated seedlings were harvested with scissors under green safe light, transferred to a stainless steel Waring blender at 4°C containing 1 L of homogenization buffer (500 mm sorbitol, 50 mm MOPS [3-(N-morpholino)propanesulfonic acid]/KOH [pH 8.0], 1 mm MgCl$_2$, and 1% [v/v] 2-mercaptoethanol) per kg tissue, and homogenized with four to five 5-second pulses on low. Homogenates were filtered through four layers of cheesecloth and two layers of Miracloth (Calbiochem, San Diego). The filtrate was centrifuged for 1 min at 7,500 rpm in a GSA rotor at 4°C. Plastid-containing pellets were gently resuspended in a total of about a one-twentieth volume (approximately 100 mL for 2 kg of oats) of ice cold homogenization buffer containing 0.2% (w/v) BSA (fraction V, heat shock) and lacking 2-mercaptoethanol. Resuspended pellets were centrifuged for 1 min at 1,000 rpm in GSA rotor. Supernatants were transferred to a fresh centrifuge bottle and centrifuged for 2 min at 4,000 rpm in a GSA rotor. The soft plastid-containing pellets were saved and the decanted supernatants were recentrifuged for 2 min at 4,000 rpm in a GSA rotor.

Etioplast pellets from the last two centrifugation steps were resuspended in ice cold plastid resuspension buffer (0.5 mm sorbitol, 100 mm potassium phosphate [pH 7.3], 2 mm EDTA, 2 mm EGTA, 1 mm phenylmethylsulfonyl fluoride, 2 mm leupeptin, and 5 mm sodium ascorbate), in a combined volume equal to 1 mL per 100 g of the original fresh weight of the tissue. Pooled etioplasts were immediately diluted into 10 volumes of ice-cold plastid lysis buffer (100 mm potassium phosphate [pH 7.3], 2 mm EDTA, 2 mm EGTA, 1 mm phenylmethylsulfonyl fluoride, 2 mm leupeptin, and 5 mm sodium ascorbate), incubated on ice with

---

**Figure 6.** Determination of kinetic constants for PΦB synthase with spinach Fd. The data were plotted and linear regression performed using Kaleidagraph 3.0. The data was plotted using the Lineweaver-Burk method. The $y$ intercept is 0.27 min AU$^{-1}$, which gives a $V_{max}$ of 3.7 AU min$^{-1}$. The $x$ intercept is $-0.24$ μm$^{-1}$, which yields an apparent $K_m$ of 4.1 μM. $R = 0.99$. **Copyright © 2001 American Society of Plant Biologists. All rights reserved.**
stirring for 10 min and ultracentrifuged at 105,000g for 1 h in a Type 35 preparative ultracentrifuge rotor at 4°C. Supernatants (i.e. soluble protein lysates) were concentrated to 0.1× to 0.2× of the starting volume (i.e. between 10 and 20 mL kg⁻¹ of starting material) using an Amicon Ultrafiltration Stirred Pressure Cell with a washed YM10 membrane.

Crystalline ammonium sulfate was added to the concentrated lysate to yield 50% saturation [291 mg (NH₄)₂SO₄ mL⁻¹] and the mixture was stirred overnight at 4°C. After centrifugation with an SS34 rotor at 10,000g for 25 min, the supernatant fraction was transferred to a fresh centrifuge tube and additional solid ammonium sulfate was added to 70% saturation [125 mg (NH₄)₂SO₄ mL⁻¹]. This mixture was incubated with stirring for several hours to overnight at 4°C and the 50% to 70% ASP was collected by centrifugation with an SS34 rotor at 10,000g for 25 min. The well-drained 50% to 70% ASP was dissolved in 2 mL of 20 mm TEA/KOH (pH 7.5) per kg of starting material, transferred to a 12,000 to 16,000 MWCO dialysis bag, and dialyzed overnight against 100 volumes of 20 mm TEA/KOH and 60 mm KCl (pH 7.5).

Dialyzed 50% to 70% ASP fractions were loaded onto a Pharmacia THC system consisting of three different 1-ml Pharmacia HiTrap cartridges, HiTrapQ, HiTrapSP, and HiTrapBlue, that had been pre-equilibrated with 20 mm TEA/HCl-KOH and 60 mm KCl (pH 7.5) using a Dionex BioLC. The flow rate for all pre-equilibration, loading, washing, and elution was 1 mL min⁻¹. The tandem HiTrap column setup was washed with five to 10 volumes of equilibration buffer until the baseline was stabilized, at which point the column setup was disassembled and the HiTrapBlue cartridge reconnected. The HiTrapBlue cartridge was eluted with a 10-mL linear gradient of 0.06 to 1 M KCl in 20 mm TEA/HCl-KOH, pH 7.5 (10 mL) followed by a 5-mL linear gradient of 1 to 2 M KCl in 20 mm TEA/HCl-KOH, pH 7.5. Fractions (1 mL) were collected and assayed for PΦB synthase activity using the coupled assay described below, and the active fractions were pooled.

Pooled HiTrapBlue fractions were buffer exchanged with 10 mM potassium phosphate, 10% (v/v) glycerol (pH 7.3) using a Millipore Ultrafree 4 centrifuge filter with a BioMax 10 kD NMWCO membrane. The desalted fraction was then loaded onto a 1-ml Bio-Rad EconoPac CHT-II hydroxyapatite cartridge that was precharged with 400 mM potassium phosphate, 10% (v/v) glycerol (pH 7.3), and equilibrated with 10 mM potassium phosphate, 10% (v/v) glycerol (pH 7.3). The column was eluted with a 10-volume linear gradient of 10 to 400 mM potassium phosphate buffer (pH 7.3). The flow rate was 0.5 mL min⁻¹ and 0.5-mL fractions were collected. Column fractions were assayed for PΦB synthase activity using the coupled assay described below, and the active fractions were pooled.

The CHT-II fraction was concentrated to less than 50 μL using a Millipore Ultrafree 4 centrifuge filter with a BioMax 10-kD NMWCO membrane. The concentrated fraction was chromatographed at 4°C on a Pharmacia SMART system using a Superdex 200 3.2/30-gel filtration column previously equilibrated with 25 mm TES/KOH, 100 mM KCl, and 10% (v/v) glycerol (pH 7.3) at a flow rate of 30 μL min⁻¹. The column eluant was monitored at 280 nm. Column fractions were assayed for PΦB synthase activity using the coupled assay. For determination of the relative molecular mass of PΦB synthase, Bio-Rad molecular mass standards were used.

Gel filtration column fractions exhibiting the highest levels of activity were pooled and loaded onto non-denaturing Pharmacia IEF PHAST gels (pH 6.5–3.0) and focused with a Pharmacia PHAST system. The focused gel was sliced into 2-mm fractions for an activity measurement using the coupled assay or stained with either silver or acid violet 17 (see below). After staining, the target proteins were excised from the gel and transferred to a 1.5-mL microcentrifuge tube. An equal volume of 2× SDS-PAGE sample buffer was added to the gel fragments, and the sample heated to 95°C for 2 min. The IEF fractions then were loaded into wells of a 12.5% (w/v) SDS-PAGE gel, electrophoresed, and the gel stained with Coomassie Brilliant Blue R-250.

**PΦB Synthase Assay**

For a 1-mL assay, the protein fraction to be assayed (10–100 μL) was diluted into 50 mM TES/KOH (pH 7.3) containing an NADPH regenerating system (6.5 mM G6-phosphate, 0.82 mM NADP⁺, and 1.1 units mL⁻¹ G6-phosphate dehydrogenase-Type XII from Torula yeast [EC 1.1.1.49]), an Fd-reducing system (4.6 μM spinach Fd and 0.025 units mL⁻¹ spinach FNR [EC 1.18.1.2]) and 10 μM bovine serum albumin (fraction V, heat shock). Reactions were initiated by addition of 10 μL BV in Me₂SO to yield a final BV concentration in the assay of 5 μM. Assays were incubated in a 28°C water bath under green safe light or subdued room light for 30 min or as noted. Assays were stopped by placing them on ice. For assays of intact plastid fractions or membrane fractions, assays were clarified by centrifugation at 12,000g for 15 min at 4°C prior to workup. PΦB synthase assays mixtures were analyzed either quantitatively using HPLC or qualitatively following addition of recombinant apophytochrome and difference spectros-copy (see below).

**HPLC Analysis**

For quantitative analysis of PΦB synthase activity, assay mixtures were loaded onto a Waters C₁₈ Sep-Pak Light cartridge that had been preconditioned with 3 mL of acetoni-trile, 3 mL of MilliQ water, and 3 mL of 50 mM 4-methylmorpholine-acetic acid (pH 7.7). After the sample loading, the Sep-Pak was washed with 3 mL of 4-methylmorpholine/glacial acetic acid (pH 7.7) followed by 3 mL of 0.1% (v/v) trifluoroacetic acid in water. The Sep-Pak then was eluted with 2 mL of acetoni-trile. The eluate was dried down using a Speed-Vac lyophilizer, dissolved in 5 μL of Me₂SO, and diluted with 200 μL of the HPLC mobile phase, acetone:20 mM formic acid::50:50. The resulting solutions were centrifuged and the supernatants filtered through a 0.45 μm polytetrafluoroethylene syringe filter.
prior to reversed phase HPLC using a Varian 5000 liquid chromatograph. Samples were applied to a Phenomenex Ultragel 500 (20) 4.6-mm × 250-mm analytical column with a 4.6-mm × 30-mm guard column using an isocratic mobile phase (acetonitrile-formic acid::50:50) with a flow rate of 0.8 mL min⁻¹ and monitored at 380 nm using a Varian UV/100 flow-through absorbance detector. Peak areas were quantified using a Hewlett-Packard 3365 Chemstation II.

Coupled Assay Analysis

In some cases, a coupled assay was used as an alternative to the HPLC analysis of PFB synthase. As outlined by Terry and Lagarias (1991), an aliquot of recombinant Cph1 apophytochrome from Synechocystis PCC 6803 (Yeh et al., 1997) was added to the crude PFB synthase assay mixtures. After incubation for 20 to 30 min at room temperature under green safe light, a difference spectrum was taken as described previously to detect the presence of holophytochrome (Litts et al., 1983).

Protein Assay and Electrophoresis

Protein concentrations were determined using the Bradford protein assay with BSA as a standard (Bradford, 1976). SDS-PAGE gels were electrophoresed (Laemmli, 1970) and stained either with Coomassie Brilliant Blue R-250 or silver (Blum et al., 1987) with the modifications described in by Ausubel et al. (1991). IEF gels were stained with either silver or with acid violet 17 (Patestos et al., 1988).

ACKNOWLEDGMENTS

We thank Drs. Beronda Montgomery and Nicole Frankenberg for critical reading of the manuscript; Dr. Nick Marshall, K.C. McFarland, and Dr. Tom Berkelman for helpful scientific discussions and technical assistance; and Dr. Richard Malkin for supplying pure spinach Fd.

Received December 22, 2000; returned for revision March 8, 2001; accepted April 25, 2001.

LITERATURE CITED


Beale SI, Cornejo J (1991b) Biosynthesis of phycobilins: 3(Z)-phyceroerythrobilin and 3(Z)-phycocyanobilin are intermediates in the formation of 3(E)-phycocyanobilin from biliverdin IXa. J Biol Chem 266: 22333–22340

Beale SI, Cornejo J (1991c) Biosynthesis of phycobilins: 15,16-dihydrobiliverdin IXa is a partially reduced intermediate in the formation of phycobilins from biliverdin IXa. J Biol Chem 266: 22341–22345


Muramoto T, Kohchi T, Yokota A, Hwang IH, Goodman HM (1999) The Arabidopsis photomorphogenic mutant hyl1 is deficient in phytocrome chromophore biosynthe-
sis as a result of a mutation in a plastid heme oxygenase.
Plant Cell 11: 335–347


