NADPH:protochlorophyllide oxidoreductase (POR; EC 1.1.33.1) is a key enzyme for the light-induced greening of angiosperms. In barley, two POR proteins exist, termed PORA and PORB. These have previously been proposed to form higher molecular weight light-harvesting complexes in the prolamellar body of etioplasts (Reinbothe, C., Lebedev, N., and Reinbothe, S. (1999) *Nature* 397, 80–84). Here we report the *in vitro* reconstitution of such complexes from chemically synthesized protochlorophyllides (Pchlide) a and b and galacto- and sulfolipids. Low temperature (77 K) fluorescence measurements revealed that the reconstituted, lipid-containing complex displayed the same characteristics of photoactive Pchlide 650/657 as the presumed native complex in the prolamellar body. Moreover, Pchlide F650/657 was converted to chlorophyllide (Chlide) 684/690 upon illumination of the reconstituted complex with a 1 ms flash of white light. Identification and quantification of acetone-extractable pigments revealed that only the PORB-bound Pchlide a had been photoactive and was converted to Chlide a, whereas Pchlide b bound to the PORA remained photoinactive. Nondenaturing PAGE of the reconstituted Pchlide a/b-containing complex further demonstrated a size similar to that of the presumed native complex *in vivo*, suggesting that both complexes may be identical.

NADPH:protochlorophyllide oxidoreductase (POR)1 is a key enzyme for the light-induced greening of etiolated angiosperm plants. It catalyzes the only known light-dependent step of chlorophyll biosynthesis, the reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) (1–3). In barley, two POR proteins have been identified, termed PORA and PORB (4). Both are light- and NADPH-dependent enzymes, which differ remarkably in their expression patterns during plant development. PORA appears only transiently in dark-grown seedlings, whereas PORB is expressed in etiolated, illuminated, and light-adapted plants (4). The partial overlap in expression suggests that both PORA and PORB may be needed for efficient seedling de-etiolation. We propose that in the prolamellar body of etioplasts, the PORA and PORB may cooperate in terms of a novel “light-harvesting POR-Pchlide a/b” complex termed LHPP (5).

*In vitro* reconstitution experiments with synthetic zinc analogs of Pchlide a and Chlide b, termed zinc protopheophorbides (ZnPP) a and b, respectively, indeed supported such a model. PORA-ZnPPb-NADPH and PORB-ZnPPa-NADPH ternary complexes were found to form oligomers (5). We observed that light, which was absorbed by ZnPPb, was transferred onto ZnPPa (5). This, by virtue of PORB, was reduced to zinc pheophorbide a (5), the zinc analog of Chlide a (6). The existence of analogous higher molecular weight light harvesting structures *in vivo* was inferred from previously reported energy transfer reactions, taking place from so-called photoactive Pchlide to photoactive Pchlide and from photoactive Pchlide to Chlide, in prolamellar bodies before and after flash light illumination (7–12).

Previous critiques questioned the existence of a Pchlide a/b-containing light-harvesting complex *in vivo*, based on the following main arguments (13). First, previous work seemed to indicate a lack of Pchlide b in etiolated plants (14). Second, respective *in vitro* reconstitution experiments had thus far not been presented for Pchlide a and Pchlide b, which, according to the LHPP model (5), should be cognate substrates of the PORB and PORA, respectively. Third, neither the reconstituted nor the presumed authentic complex had been resolved under native conditions as higher molecular weight, lipid-containing structures.

In the present study, we addressed these important questions and performed *in vitro* reconstitution experiments. We demonstrate that the PORA and PORB display the same stringent substrate specificities for Pchlide a and Pchlide b as those reported previously for ZnPPa and ZnPPb (5). We further show that reconstituted PORa-Pchlide b-NADPH and PORb-Pchlide a-NADPH ternary complexes establish higher molecular mass structures, the spectroscopic and physicochemical properties of which are very close, in most aspects even indistinguishable, from those of the presumed native complex.

**MATERIALS AND METHODS**

**Cloning Procedures—**Double-stranded DNAs encoding the mature parts of the PORA and PORB proteins of barley were generated by a polymerase chain reaction-based approach (15). The following primer pairs were used: Primer 1 (5'-AAGTCGCGCTGGACAGAAGAGCTG-3') plus 2 (5'-AAGTCGCGCTGGACAGAAGAGCTG-3'), and primers 3 (5'-AAGTCGCGCTGGACAGAAGAGCTG-3') plus 4 (5'-AAGTCGCGCTGGACAGAAGAGCTG-3'), as well as cDNA clones A7 (16) and L2 (4), respectively, as templates. After subcloning into the *Pst*I site of pUC19 (New England Biolabs), the DNAs for PORA and PORB were cut out with *Bam*HI and
**In Vitro Reconstitution of LHPP with Pchlide a and b**

HindIII and inserted into identically treated pSP64 vectors (Promega) (17). The identity of the different clones was confirmed by DNA sequencing, using a T7 DNA sequencing kit (Promega) and the gel system described in Ref. 18.

**Preparation of Pigments**—Chemical synthesis, purification, and characterization of zinc- and magnesium-Pchlide a and b were performed as described in Refs. 6 and 14. HPLC was carried out either on a C18 reverse-phase silica gel column (250 × 4.6 mm, Nucleosil ODS 5 μm; Macherey-Nagel Co.) or a C30 reverse phase column (250 × 4.6 mm, 5 μm; YMC Inc., Wilmington, NC) (19), using a Varian ProStar model 410 apparatus, a ProStar model 240 pump, and a ProStar 320 photodiode array detector. In some experiments, a C18 reverse phase silica gel column (250 × 4.6 mm, Hypersil ODS 5 μm; Hypurity™), a Dynamax absorbance detector model UV-1, and a Dynamax SD-200 pump were used.

**In Vitro Transcription/Translation and Reconstitution of POR-Pigment Complexes**—Radiolabeled PORA and PORB molecules were synthesized by coupled in vitro transcription/translation (20) of the recombinant clones specified above and purified as described previously (3). Equal amounts of the PORA and PORB, as determined by counting their radioactivities and correcting the rates of incorporation for the different methionine contents (21), were supplemented with NADPH (0.5 mM final concentration) and synthetic Pchlide a, Pchlide b, ZnPps, or ZnPp6. In all cases, 10 μl of final porphyrin concentrations were used. After a 15-min incubation in the dark, the assay mixtures were subjected to gel filtration on Sephadex G15 equilibrated in assay buffer (22). Enzyme-pigment complexes eluted with the flow-through were extracted with acetone (see below), and pigments were quantified in a spectrometer LS50B (PerkinElmer Life Sciences) (23).

For the reconstitution of LHPP, equimolar amounts of the recombinant PORA-Pchlide b-NADPH and PORB-Pchlide a-NADPH ternary complexes were incubated in the dark, as described previously (3). Then the resulting high molecular weight complexes were separated from free, nonassembled POR-pigment-NADPH complexes by gel filtration on Sephadex G100 (5) or Superose 6 (Amersham Biosciences). Fractions containing PORA-PORB supercomplexes were identified by radioactivity measurements, pooled and in turn supplemented with a mixture of galactolipids containing palmitolyl(diacylglycerol)+galactosyl diacylglycerol and sulfoquinovosyl diacylglycerol (55:36:6 mol %; see Ref. 5). The sample was then cooled to −196 °C and analyzed by fluorescence emission measurements at an excitation wavelength of 440 nm. For flash light experiments, the sample was warmed to −25 °C (24), exposed to a single, 1-ms flash of white light, and immediately dipped into liquid nitrogen. Then the spectroscopic measurements were repeated. For the experiment described in the legend to Fig. 8, two parallel samples were prepared, of which one was exposed to flash light as above, whereas the other was kept in darkness before nonadenaturating electrophoresis (25).

**Protein Analysis**—Three different methods were employed to prepare and purify POR-pigment complexes, that of Ryberg and Sundqvist (26), that of Klement et al. (27), and a modified version of that of Gerhardt and Heldt (28).

In the first case, etioplasts were isolated from 5-day-old dark-grown barley plants by differential centrifugation (for details, see Ref. 26). One aliquot of the final etioplast suspension was extracted with an excess of cold acetone containing 0.1% (v/v) diethyl pyrocarbonate, and protein aliquot of the final etioplast suspension was extracted with an excess of cold acetone containing 0.1% (v/v) diethyl pyrocarbonate, and protein was recovered, diluted, and resedimented by centrifugation. The resulting etioplast pellet was resuspended in a buffer containing 50 mM Tricine/KOH, pH 7.2, 20% glycerol, and four equal parts were loaded onto sucrose buffer containing 0.5 mM sucrose, 1 mM MgCl2, 1 mM EDTA, 10 mM Tricine, 10 mM HEPES, pH 7.2. After a step of ultracentrifugation at 80,000 × g for 20 min, the resulting pellet was resuspended in the same buffer as described before but containing 2.5 mM n-ocyt-β-glucoside and 5% glycerol. After gentle shaking for 30 min, the assays were reincubated as described previously, and the obtained pellet was treated with the same detergent buffer, but containing 15 mM n-ocyt-β-glucoside and 30% glycerol. The suspension was again gently shaken for 30 min and subjected to ultracentrifugation at 200,000 × g for 20 min. The resulting supernatant was loaded onto a column (9 × 32 mm) of DEAE-cellulose (Sigma) equilibrated with a buffer containing 5 mM MgCl2, 0.3 mM n-ocyt-β-glucoside, and 10 mM Tricine, 10 mM HEPES, pH 7.2. Protein was eluted from the column by applying 12 ml of a gradient consisting of 5–20 mM n-ocyt-β-glucoside dissolved in equilibration buffer. Fractions of 0.5 ml were taken and analyzed for the presence of POR by Western blotting with the PORA antiserum described before.

As the third method, we adopted the nonaqueous fractionation technique of Gerhardt and Heldt (28) to isolated intact etioplasts. Briefly, etioplasts were isolated by differential centrifugation and then loaded onto a density gradient centrifugation and further purified on cushions of Percoll as described (30). Each plastid sample was divided into two equal parts, of which one was exposed to a single flash of white light, whereas the other was kept in darkness. Etioplasts were then resedimented and immediately quenched and ground under liquid nitrogen. The etioplast powder was then lyophilized at −50 °C. About 200–300 mg of the dry plastid material were transferred at −35 °C into a mixture of heptane/ carbon tetrachloride (C7H16/CCl4 66:34 (v/v), density 1.28 g/cm3). The suspension was in turn ultrasonicated at −70 °C with 10 s–5 s pulses in a Branson Sonifier (see above) and poured through a layer of quartz wool contained in a filter to remove any remaining coarse material. The flow-through was diluted 3-fold with heptane and centrifuged for 2 min at 3000 × g. The clear supernatant was discarded, and the sediment was resuspended in 3 ml of a CCl4/C3H8 mixture of the same density as described above. Two 200-μl aliquots were withdrawn for determination of Pchlide a and Pchlide b levels by HPLC and enzyme activities (see below). The remainder was loaded onto a freshly prepared, exponential 1.25–1.50 g/cm2 density gradient of CCl4/C3H8. After centrifugation at 25,000 × g for 2.5 h, during which time the material distributed isopycnically in the gradient, 1.2 ml fractions were removed, starting from the top of the gradient, and subsequently divided into three equal portions. One-third was used for determination of marker enzymes (NADPH-glycereraldehyde-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, vacuole), the second was used for SDS-PAGE, and the third portion was used for assay of Pchlide a and Pchlide b levels. All three divided portions and the two aliquots taken from the original sample (see above) were diluted 3-fold with heptane and centrifuged for 8 min at 18,000 × g. The supernatant was discarded, except for the last 200 μl, which were used to reusenad the sediment by swirling it with a calcined quartz. All samples were then dried for 18 h in a desiccator and then processed for electrophoresis, enzyme assays, or pigments measurements. All manipulations were performed under a dim green light. Moreover, any step that was expected to potentially lead to trapping of condensing water vapor was carefully avoided (28).

**Electrophoresis—SDS-PAGE was performed in 10–20% (w/v) gradients of polyacrylamide as described (31). Nonadennaturating, analytical PAGE was performed in 5-mm-thick 7.5% (w/v) polyacrylamide gels, and the gels were run using a discontinuous buffer system (25).
RESULTS

Our previous reconstitution experiments had shown that the PORA and PORB proteins of barley are able to form higher molecular weight light-harvesting complexes if complexed with ZnPP\textsuperscript{b} and ZnPP\textsuperscript{a}, respectively, plus NADPH (5). As a first step to establish such complexes also with their presumed natural substrates, we synthesized Pchlide\textsubscript{a} and Pchlide\textsubscript{b} chemically (6, 14). The isolated and purified pigments are characterized in an accompanying paper (32). They were added to PORA and PORB polypeptides, which had been synthesized from corresponding cDNA clones by coupled transcription/translation of respective recombinant clones and purified, and 1, 0.2, 0.1, and 0.02 POR protein equivalents were subsequently incubated for 15 min in the dark with Pchlide\textsubscript{a} (Δ——Δ), Pchlide\textsubscript{b} (▲——▲), ZnPP\textsuperscript{b} (□——□), and ZnPP\textsuperscript{a} (■——■), respectively. After a step of gel filtration on Sephadex G15, POR-bound pigments were extracted with a solution of practically pure acetone containing 0.1% (v/v) diethyl pyrocarbonate. Pigments were identified and quantified by either HPLC analyses and subsequent absorbance measurements or by fluorescence emission coefficients of isolated pigments (6, 14). The plots show the amounts of PORA-bound and PORB-bound pigments versus the PORA and PORB protein concentrations in the assays. 1 POR protein equivalent corresponded to 2.78 pmol of the PORA and 2.64 pmol of the PORB, respectively.

Fig. 1 shows a plot of the amount of Pchlide\textsubscript{a} and Pchlide\textsubscript{b} bound to the PORA or PORB versus the enzyme concentrations. From the linear relationships, it turned out that 1 μg of the PORA bound ~34.11 ng of Pchlide\textsubscript{a} and 31.95 ng of ZnPP\textsubscript{b} but only 3.5 ng of Pchlide\textsubscript{b} and 3.28 ng of ZnPP\textsubscript{a}, respectively. This corresponded to 27.72 pmol of PORA, 54.3 pmol of Pchlide\textsubscript{b} (ZnPP\textsubscript{b}), and 5.7 pmol of Pchlide\textsubscript{a} (ZnPP\textsubscript{a}), respectively, and suggested that there were 1:2 versus 1:0.2 stoichiometries of PORA to pigment in the recovered POR-Pchlide (ZnPP) complexes. For the PORB, just the opposite binding preferences were seen: 1 μg (26.26 pmol) of the PORB bound ~33.2 ng of Pchlide\textsubscript{a} (31.1 ng of ZnPP\textsubscript{a})(each corresponding to ~54.2 pmol of the pigment) and only 3.2 ng of Pchlide\textsubscript{b} (3.02 ng of ZnPP\textsubscript{b}) (each corresponding to ~5.1 pmol of the pigment).

Given that the PORA and PORB displayed the same stringent substrate specificities for Pchlide\textsubscript{a} and \textsubscript{b} as those reported previously for ZnPP\textsubscript{a} and ZnPP\textsubscript{b} (5), we next established reaction conditions that would allow the generation of higher molecular weight Pchlide/por-POR-light-harvesting complexes. Equimolar amounts of reconstituted PORA-Pchlide\textsubscript{a}/NADPH and PORB-Pchlide\textsubscript{b}/NADPH ternary complexes were mixed and, after a 15-min incubation in darkness, subjected to a further step of gel filtration on Sephadex G100 (5) or size-fractionated on a Superose 6 column (see “Materials and Methods”).

Fig. 2A shows a size fractionation on Superose 6. It revealed that the pigment-complexed PORA and PORB indeed gave rise to a higher molecular weight complex. Its size of ~480 kDa was similar to that of the so-called Pchlide holochrome of bean (33). Free, nonassembled PORA- and PORB-pigment ternary complexes were eluted at much later time points (Fig. 2A).

To determine the stoichiometry of the PORA and PORB in the recovered supracomplexes, fractions containing the different PORs were pooled (see Fig. 2A) and separated by SDS-PAGE, and the gel was subjected to autoradiography. This experiment revealed that in the recovered higher molecular weight complex about five PORA-Pchlide\textsubscript{a}/NADPH ternary complexes interacted with just one PORB-Pchlide\textsubscript{b}/NADPH complex (Fig. 2B, fraction 5).

The oligomeric PORA-PORB protein complex contained in fraction 5 was subsequently supplemented with a lipid mixture containing monogalactosyl diacylglycerol, digalactosyl diacylglycerol, and sulfoquinovosyl diacylglycerol (58:36:6 mol %), which had been prepared from pigment-free prolamellar bodies of barley etioplasts (5). Then low temperature measurements were performed at 77 K, in a PerkinElmer Life Sciences spectrometer LS50B (23).

Fig. 2C (solid line) demonstrates that two fluorescence peaks could be seen at an excitation wavelength of 440 nm: one at 657 nm and the other at 632 nm. Because these two peaks corresponded to photoactive Pchlide 650/657 and photoinactive Pchlide F628/632, known from the prolamellar body of etioplasts (19), a C30 column was used. Synthetic Chlides \textsubscript{a} and \textsubscript{b} were prepared by the chlorophyllase reaction (34) and used as standards. Fig. 3B (panel c) shows that Chlide\textsubscript{a} and Chlide\textsubscript{b} were well resolved on the C30 column and also sepa-
rated from Chl a and Chl b. When the pigments, eluting at ~14 min on the C18 column (see Fig. 3A), were applied to the C30 column, the only detectable pigment was Chlide a (Fig. 3B, panel d). Thus, only Chlide a had been produced upon flash light illumination of the reconstituted complex (Table I). By contrast, Pchlide b, which was ~5-fold more abundant than Pchlide a, did not seem to be photoconvertible at all under the tested conditions. Indeed, no Chlide b was formed (Table I).

An explanation for these findings could be that the PORA was per se inactive with Pchlide b and thus unable to convert the pigment to Chlide b. If so, no Chlide b should be produced also in situ. To test this hypothesis, we first analyzed pigments that were formed in isolated prolamellar bodies upon flash light illumination by HPLC. Fig. 4 shows a representative separation of pigments before (A) and after (B) a saturating 1-ms flash of white light. In addition to Pchlide b and Pchlide a, eluting at 12.5 and 15 min, respectively (Fig. 4A), 7-hydroxy-Pchlide a could also be detected (peak 1) (for details, see Ref. 32). Upon flashing the sample, a novel pigment peak appeared (Fig. 4B, peak 4), the retention time and absorption properties of which were indistinguishable from those of Chlide a identified previously (data not shown, but see Fig. 3). At the same time, increasing amounts of Pchlide a (peak 3) were detectable.

As shown in an accompanying paper (32), barley etioplasts contain an enzyme called 7-formyl reductase that converts Pchlide b to Pchlide a via 7-hydroxy-Pchlide a (see also Ref. 14). Upon resolution of pigments contained in peak 4 by subsequent HPLC on a C30 column, indeed only Chlide a and no Chlide b was observed (data not shown).

As a second approach, the in vitro synthesized PORA was incubated with ZnPPb (which is chemically more inert than Pchlide b) and separated from nonbound pigment by gel filtra-
In Vitro Reconstitution of LHPP with Pchlide a and b

Quantification of pigments before and after flash light illumination of the reconstituted, lipid-containing complex

Pigments were extracted from the flashed and nonflashed sample with 100% acetone containing 0.1% (v/v) DEP and separated and identified by HPLC as described under “Materials and Methods.” Pigment levels refer to the sum of all detected pigments, set as 100. ND, non-detectable pigment levels.

| Pigment   | Pigment (percentage of total) |
|-----------|-------------------------------|
|           | Nonflashed | Flashed |
| Pchlide a | 22          | 4       |
| Pchlide b | 78          | 78      |
| Chlide a  | ND          | 20      |
| Chlide b  | ND          | ND      |

Fig. 4. Photoconversion of Pchlide a, but not Pchlide b, in isolated prolamellar bodies. Prolamellar bodies were isolated from barley etioplasts and either kept in darkness (A) or exposed to a 1-ms flash of white light (B). Pigments were separated by HPLC as described in Fig. 3A and identified using synthetic 7-hydroxy-Pchlide a (peak 1), Pchlide b (peak 2), Pchlide a (peak 3), and Chlide a (peak 4) as standards (not shown, but see Ref. 32). Note the limited amount of Chlide a in the illuminated sample and the relative increase in the level of Pchlide a, which is indicative of 7-formyl reductase activity present in barley etioplasts (see also an accompanying paper (32)).

All results presented thus far implied that the reconstituted, lipid-containing Pchlide a/6-POB protein complex may be identical with the presumed native complex in the prolamellar body of etioplasts. However, except for the spectroscopic data, no other line of evidence seemed to exist to support this notion. We consequently sought to identify the native complex by classical biochemical approaches and to compare its properties with those of the reconstituted complex.

Ryberg and Sundqvist (26) had shown that isolated etioplast inner membranes from wheat can be resolved into different subfractions, designated prolamellar bodies and prothylakoids, respectively, based on their different buoyant densities in sucrose gradients. We readdressed this previous work for barley etioplasts and analyzed the abundance of the PORA and PORB after various steps of the plastid work-up procedure (see “Materials and Methods”). As shown in Fig. 6, already during the very first step of isolation of the so-called etioplast inner membranes (presumed to comprise prolamellar bodies and prothylakoids) (26), a major part of the PORA became soluble. The same effect was seen for oat and wheat etioplasts, which were analyzed in parallel (data not given). In all cases, subsequent steps of prolamellar body and prothylakoid separation turned out to correlate with a further solubilization of the PORA. This is shown for barley in Fig. 6. With etioplast inner membranes, which had been diluted with a buffer lacking any additives for membrane stabilization, the PORA was quantitatively released.

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and then co-migrated in the uppermost fractions of a top-loaded 10–50% (w/w) sucrose density gradient. With barley etioplast inner membranes, which had been sonicated in a buffer containing 50% sucrose and loaded from the bottom, both the PORA and PORB appeared to be recovered in the lower parts of the gradient, containing prolaminellar bodies. However, the approximately equimolar amounts of the PORA and PORB, which were at variance with their original stoichiometries in intact etioplasts, and the fact that both POR proteins migrated to slightly different positions in the gradient argued against working further with these samples.

In a recent paper, Klement et al. (27) reported the isolation of a pigment-free POR protein from oat. Such a preparation seemed particularly interesting to us because it would allow testing of the pigment binding properties of the PORA and PORB in a more natural environment than in the in vitro system. In principal, the method of Klement et al. (27) employs differential detergent solubilization of POR from the prolaminellar body.

When we reproduced the published protocol and followed the fate of the PORA and PORB by Western blotting (Fig. 7), again drastic losses of the PORA during successive steps of the membrane preparation and solubilization procedure became apparent. In the final supernatant, approximately equal levels of the PORA and PORB were seen, which were at variance with the determined 5:1 protein stoichiometry in intact etioplasts. Moreover, subsequent chromatography on DEAE-cellulose gave rise to at least three different POR protein bands, representing the PORA, the PORB, and a slightly smaller degradation product (Fig. 7). To what extent these proteins would contribute to pigment binding could not be estimated.

Given this uncertainty and the fact that neither the method of Ryberg and Sundqvist (26) nor that of Klement et al. (27)
TABLE II
Quantification of POR and pigment levels in isolated etioplasts before and after flash light illumination

Proteins and pigments were extracted from lyophilized etioplasts before and after flash light illumination and analyzed by SDS-PAGE and HPLC, respectively, as described under "Materials and Methods." Levels are given in percentage of total, set as 100. ND, nondetectable levels.

| Pigment | Sample | Nonflashed | Flashed |
|---------|--------|------------|---------|
| Pchlide a | | 18 | ND |
| Pchlide b | | 82 | 82 |
| Chlide a | | ND | 18 |
| Chlide b | | ND | ND |
| POR | | 81 | 81 |
| PORB | | 19 | 19 |

allowed the recovery of an intact PORA-PORB protein complex, we sought alternative methods. Taking into account a paper of Gerhardt and Heldt (28) on enzyme and metabolite measurements in different subcellular compartments, we adapted nonaqueous protein and pigment extraction and fractionation to isolated etioplasts. The method employed is based on the fact that the proteins and metabolites of a given compartment aggregate together upon lyophilization. Because each compartment has a characteristic protein, lipid, carbohydrate, and ion complement, different fractions are obtained in nonaqueous gradients of carbon tetrachloride/heptane. These and an original etioplast sample were analyzed with respect to the PORA and PORB protein abundances as well as Pchlide a and Pchlide b levels. Moreover, we used nondenaturing, analytical PAGE (25) to directly visualize POR-pigment complexes in etioplasts prior to fractionation.

Table II shows the determined PORA and PORB as well as Pchlide a and Pchlide b levels. It turned out that PORA is ~4.2-fold more abundant in amount than PORB. Quantification of pigments showed that etioplasts contain an ~4.5-fold excess of Pchlide b relative to Pchlide a. Of the total Pchlide, only 18% was photoreducible. This photoconvertible Pchlide turned out to be identical with Pchlide a (Table II).

Fig. 8A (panel a, lane D) shows a nondenaturing, analytical PAGE of the presumed natural POR-pigment complex. Based on the red light-induced autofluorescence of Pchlide F650/657, this complex could directly be visualized by fluorography. Western blot analyses confirmed that it contained POR (Fig. 8A, panel b, lane D). Flash light illumination and subsequent mild detergent treatment in the presence of 0.2% (v/v) Triton X-100 prior to electrophoresis dissociated this total POR into two subfractions (Fig. 8A, FL). Upon scaling up the procedure 1000-fold, these could be identified as PORA and PORB by protein sequencing (data not shown). Their approximate sto-
In Vitro Reconstitution of LHPP with Pchlides a and b

chemistry was similar to that determined from the carbon tetrachloride/heptane gradients (Table II) and also matched that expected from our previous in vitro reconstitution experiments (Fig. 2). Indeed, when the in vitro reconstituted, lipid-containing complex was subjected to non-denaturing PAGE, a similar, although slightly smaller, complex could be seen (Fig. 5D). This complex contained both PORA and PORB and displayed the same type of autofluorescence as the presumed native complex. Moreover, it was rapidly dissociated into the two POR proteins upon flash light illumination.

An HPLC analyses of pigments reextracted from the electrophoretically resolved native POR-pigment complex is shown in Fig. 9. It demonstrated that the complex contained both Pchlide b and Pchlide a (Fig. 9A, peak 2 and 3, respectively). In addition, substantial amounts of 7-hydroxy-Pchlide a could also be seen (Fig. 9A, peak 1). Upon flash light illumination, correlating with the disintegration of the complex (see Fig. 8A), only Chlide a was produced (Fig. 9B, peak 4). It co-migrated with the PORB protein band (Fig. 9D). Protochlorophyllide b, by contrast, remained quantitatively unchanged (Fig. 9, compare B versus A) and co-migrated with the PORB protein band (Fig. 9C). This result not only confirmed the previously determined substrate specificities but also that only PORB’s bound pigment (i.e. Pchlide a) had been converted to Chlide a. The bulk of the pigment, corresponding to Pchlide b, remained non-photoconvertible.

**DISCUSSION**

In the present study, we addressed three different questions. First, would the PORA and PORB display the same stringent light-harvesting structures with galacto- and sulfolipids, as also be seen (Fig. 9B, peak 4)? Second, would the resulting PORA-Pchlide b-NADPH and PORB-Pchlide a-NADPH ternary complexes be able to establish higher molecular weight light-harvesting structures with galacto- and sulfolipids, as proposed previously (5)? Third, would similar, Pchlide b-containing complexes exist in vivo?

The answers to all of these questions were positive. We were able to demonstrate that PORB binds ~10-fold higher amounts of Pchlide b (ZnPPb) relative to Pchlide a (ZnPPa). PORB, by contrast, was specific for Pchlide a (ZnPPa) and bound ~10-fold lower levels of Pchlide b (ZnPPb). Either POR protein likewise converted these compounds into their respective products in vitro. However, if PORA-Pchlide b-NADPH and PORB-Pchlide a-NADPH ternary complexes were mixed and reconstituted to higher molecular weight complexes, only the PORB remained active. In the presence of galacto- and sulfolipids, the reconstituted Pchlide a/b-POR complex displayed the features of Pchlide F650/657. This Pchlide F650/657 was converted to Chlide F684/690 upon flash light illumination. Indistinguishable spectral pigment species and pigment conversions have been described for isolated prolamellar body membranes of etioplasts (see Introduction). Moreover, we were able to resolve the lipid-containing structure both from the prolamellar body and after in vitro reconstitution into similar higher molecular weight complexes under native conditions. Based on all of these findings, we believe that the reconstituted and analyzed authentic complexes may be structurally and functionally identical.

How is LHPP made in vivo? A key aspect related to this question refers to the origin of Pchlide b. The existence of this pigment has long been a matter of dispute (see literature cited in Ref. 14). As shown in this and an accompanying paper (32), the pigment is present in etiolated barley plants but is rapidly converted to Pchlide a if no precautions are taken. 7-Formyl reductase presumably responsible for this conversion is highly active in etioplasts. It was for a long time implicated in Chl b to Chl a conversion, but it appears that 7-formyl reductase plays a more general role in fine tuning the levels of both porphyrins and chlorins in dark-grown and illuminated plants (14, 32).

Enzymes, which may synthesize Pchlide b, have not been identified. The most likely candidates are proteins that could display (P)Chlide a oxygenase activity. Previous work has shown that there is a family of related proteins, which may exhibit such an activity (15-41). Tomita et al. (36) cloned a Chlamydomonas reinhardtii cDNA for a putative Chlide a oxygenase. Later studies by Espinosa et al. (37) and Tomitani et al. (38) showed that highly related Chlide a oxygenase sequences also occur in Arabidopsis thaliana and other plant species. The Arabidopsis protein was expressed in bacteria and suggested to display Chlide a, but not Pchlide a, oxygenase activity (39). Recent work by Xu et al. (40, 41), however, highlighted that heterologous expression of the Arabidopsis Chlide a oxygenase in cyanobacteria leads to Pchlide b and Chlide b accumulation. Although this demonstrates that Chlide a oxygenase is well able to bind and convert Pchlide a to Pchlide b, it is not yet known whether Chlide a oxygenase is expressed in etiolated plants. If this does not occur, another enzyme should exist that drives Pchlide b synthesis.

Recent, yet unpublished work for barley shows that there is indeed a protein related to Chlide a oxygenase (for sequence comparisons, see Refs. 35 and 36), which is able to convert Pchlide a to Pchlide b. It is part of the substrate-dependent import machinery in the plastid envelope through which the cytosolic precursor of the PORa is imported into the organelle (21, 22, 30). We were able to demonstrate that Pchlide a, formed upon feeding isolated plastids the Pchlide precursor 5-aminolevulinic acid, is converted to Pchlide b. Concomitantly, the envelope-bound PORa precursor was chased into the plastids and processed to mature size. These findings imply that Pchlide b synthesis is directly coupled to the import step and that the novel Pchlide a oxygenase is located in the plastid envelope.

Consistent with such an idea are also previous findings that isolated envelope membranes of spinach chloroplasts contain Pchlide (42, 43). Work is in progress to further characterize the novel Pchlide a oxygenase.

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