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In Vitro Reconstitution of Light-harvesting POR-Protochlorophyllide Complex with Protochlorophyllides *a* and *b**

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Christiane Reinbothe‡§, Frank Buhr‡, Stephan Pollmann¶, and Steffen Reinbothe¶‖

From the ‡Lehrstuhl für Pflanzenphysiologie, Universität Bayreuth, Universitätsstraße 30, D-95447 Bayreuth, Germany, the ¶Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität Bochum, Universitätsstraße 150, D-44801 Bochum, Germany, and the ‖Université Joseph Fourier et CNRS, UMR 5575, BP53, CERMO, F-38041 Grenoble cedex 9, France

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 (Pchlides) *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 proposed 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 Pchlide *b*, termed zinc protopheophorbides (ZnPP) *a* and *b*, respectively, indeed supported such a model. PORA-ZnPP*b*-NADPH and PORB-ZnPP*a*-NADPH ternary complexes were found to form oligomers (5). We observed that light, which was absorbed by ZnPP*b*, was transferred onto ZnPP*a* (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 photoinactive Pchlide to photoactive Pchlide and from photoinactive 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 ZnPP*a* and ZnPP*b* (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′-AACTGCAGATGGGCAAGAAGACGCTGCGGCAG-3′) plus 2 (5′-AACTGCAGGGTGGATCATAGTCCGACGAGCTT-3′), and primers 3 (5′-AACTGCAGATGGGCAAGAAGACTGTCCGCACG-3′) plus 4 (5′-AACTGCAGTGATCATGCGAGCCCGACGAGCTT-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

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§ To whom correspondence should be addressed: Lehrstuhl für Pflanzenphysiologie, Universität Bayreuth, Universitätsstr. 30, 95447 Bayreuth, Germany. Tel.: 49-921-55-26-27; Fax: 49-921-75-77-442; E-mail: christiane.reinbothe@uni-bayreuth.de.

[1] The abbreviations used are: POR, NADPH:protochlorophyllide oxidoreductase; Chl, chlorophyll; Chlide, chlorophyllide; HPLC, high performance liquid chromatography; LHPP, light-harvesting POR-Pchlide complex; Pchlide, protochlorophyllide; ZnPP, zinc protopheophorbide; TES, *N*-tris(hydroxymethyl-2-aminomethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

*Hin*dIII 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-Pchlides *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.) (6) 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 330 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*, ZnPP*a*, or ZnPP*b*. In all cases, 10 μM 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 reconstituted PORA-Pchlide *b*-NADPH and PORB-Pchlide *a*-NADPH ternary complexes were incubated in the dark, as described previously (5). 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 supracomplexes were identified by radioactivity measurements, pooled and in turn supplemented with a mixture of galacto- and sulfolipids containing monogalactosyl diacylglycerol, digalactosyl diacylglycerol, and sulfoquinovosyl diacylglycerol (58:36:6 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 about −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 nondenaturing electrophoresis (25).

Protein Analyses—Three different methods were employed to prepare and analyze 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 100% acetone containing 0.1% (v/v) diethyl pyrocarbonate, and protein was recovered by centrifugation and subsequently used for Western blot analysis (29), using an antiserum against the PORA of barley (16). In three additional samples, the plastids were lysed hypotonically in a buffer containing 1 mM $MgCl_2$, 1 mM EDTA, 20 mM TES, 10 mM HEPES, pH 7.2, and etioplast inner membranes comprising prolamellar bodies and prothylakoids prepared by homogenization in a glass homogenizer. All samples were then centrifuged at 7,700 × *g* for 15 min. For one sample, proteins found in the resulting pellet and supernatant, respectively, were extracted with acetone (see above), sedimented, and used for subsequent Western blot analysis (see above). In the case of the second sample, the obtained pellet was resuspended in the buffer described before but containing 50% sucrose and sonicated three times for 5 s each with a Branson Sonifier (Danbury, CT) (microtip, medium tune), and the homogenate was placed at the bottom of a continuous 10–50% (w/w) sucrose gradient. In case of the third sample, essentially the same procedure was followed, except for the fact that the buffer used for ultrasonication lacked sucrose and that the etioplast inner membranes were loaded from the top onto the gradient.

After centrifugation of the gradients at 25,400 × *g* for 2 h, several different bands were seen. For the bottom-loaded gradient, these corresponded to buoyant densities of ~1.17 g cm^{-3} and ~1.21 g cm^{-3}, respectively. In case of the top-loaded gradient, the lighter band was also obtained, but not the heavier. Instead a novel, diffuse band spreading over at least three fractions (designated *T1–3* in Fig. 6) was recovered in the uppermost parts of the gradient. According to Ryberg and Sundqvist (26), band T1 as well as the band at 1.21 g cm^{-3} should represent prothylakoids, whereas the 1.17 g cm^{-3} band should be identical with prolamellar bodies. All of the different bands were retrieved from the gradients, diluted 4-fold with the buffer described above, and finally centrifuged at 42,500 × *g* for 2 h. Proteins found in the resulting pellet fractions were then extracted with acetone (see above) and analyzed by Western blotting (29).

According to Klement *et al.* (27), etioplasts were prepared from etiolated barley plants by Percoll density gradient centrifugation, retrieved, 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 M sucrose, 1 mM $MgCl_2$, 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*-octyl-β-D-glucoside and 5% glycerol. After gentle shaking for 30 min, the assays were recentrifuged as described previously, and the obtained pellet was treated with the same detergent buffer, but containing 15 mM *n*-octyl-β-D-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 *n*-octyl-β-D-glucoside, 10% glycerol, 0.3 mM $MgCl_2$, 0.3 mM EDTA, 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*-octyl-β-D-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 adapted the nonaqueous fractionation technique of Gerhardt and Heldt (28) to isolated intact etioplasts. Briefly, etioplasts were isolated by differential centrifugation and Percoll 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 (C_7H_{16}/CCl_4 66:34 (v/v), density 1.28 g/cm^3). The suspension was in turn ultrasonicated at −70 °C with 10 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 CCl_4/C_7H_{16} mixture of the same density as that described before. 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 1.28–1.50 g/cm^3 density gradient of CCl_4/C_7H_{16}. 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:glycerinaldehyde-phosphate dehydrogenase, plastid stroma; phosphoenolpyruvate carboxylase, cytosol; α-mannosidase, 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 resuspend 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 pigment 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). Nondenaturing, analytical PAGE was performed in 3-mm-thick 7.5% (w/v) polyacrylamide gels, and the gels were run using a discontinuous buffer system (25).

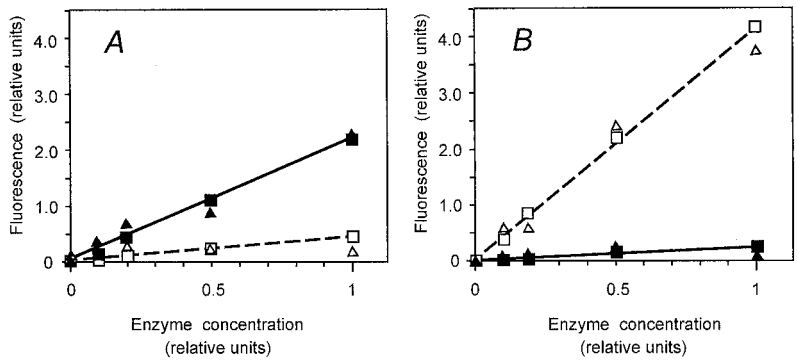

FIG. 1. **Pigment binding characteristics of the PORA (*A*) and PORB (*B*).** PORA and PORB proteins were produced by coupled *in vitro* transcription/translation of respective recombinant clones and purified, and 1, 0.2, 0.1, and 0.02 POR protein equivalents were subsequently incubated for 15 min in the dark with Pchlide *a* (△——△), Pchlide *b* (▲——▲), ZnPP*a* (□——□), and ZnPP*b* (■——■), 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 spectroscopy, taking into account previously published absorption and 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.

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*b* and ZnPP*a*, respectively, plus NADPH (5). As a first step to establish such complexes also with their presumed natural substrates, we synthesized Pchlides *a* and *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 *in vitro* transcription/translation (20). Pigment binding was tested in the following manner. Different amounts of the PORA and PORB were added to an excess of NADPH and isolated pigment, incubated for 15 min in darkness, and subsequently separated from the excess of nonbound pigments by gel filtration on Sephadex G15 (22). POR-pigment complexes running in the flow-through were then extracted with 100% acetone containing 0.1% (v/v) diethyl pyrocarbonate, and pigments were identified and quantified by HPLC and room temperature absorbance and fluorescence measurements.

Fig. 1 shows a plot of the amount of Pchlide *a* and Pchlide *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 *b* and 31.95 ng of ZnPP*b* but only 3.5 ng of Pchlide *a* and 3.28 ng of ZnPP*a*, respectively. This corresponded to 27.72 pmol of PORA, 54.3 pmol of Pchlide *b* (ZnPP*b*), and 5.7 pmol of Pchlide *a* (ZnPP*a*), respectively, and suggested that there were 1:2 *versus* 1:0.2 stoichiometries of PORA to pigment in the recovered PORA-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 *a* (31.1 ng of ZnPP*a*)(each corresponding to ~54.2 pmol of the pigment) and only 3.2 ng of Pchlide *b* (3.02 ng of ZnPP*b*) (each corresponding to ~5.1 pmol of the pigment).

Given that the PORA and PORB displayed the same stringent substrate specificities for Pchlides *a* and *b* as those reported previously for ZnPP*a* and ZnPP*b* (5), we next established reaction conditions that would allow the generation of higher molecular weight Pchlide *a*/*b*-POR light-harvesting complexes. Equimolar amounts of reconstituted PORA-Pchlide *b*-NADPH and PORB-Pchlide *a*-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. 2*A* 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. 2*A*).

To determine the stoichiometry of the PORA and PORB in the recovered supracomplexes, fractions containing the different PORs were pooled (see Fig. 2*A*) 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 *b*-NADPH ternary complexes interacted with just one PORB-Pchlide *a*-NADPH complex (Fig. 2*B*, 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. 2*C* (*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 (*e.g.* Ref. 5), we exposed the lipid-containing complex to a saturating 1-ms flash of white light. As shown in Fig. 2*C* (*dashed line*), this gave rise to the quantitative conversion of Pchlide F650/657 to Chlide 684/690.

We next extracted pigments from the flashed sample with acetone (see above) and ran HPLC analyses, as described in Ref. 14. Separation on a C18 column is shown in Fig. 3*A*. As demonstrated in an accompanying paper (32), the pigment peak eluting at ~12.5 min is identical with Pchlide *b*, whereas the peak eluting at ~15 min is identical with Pchlide *a* (Fig. 3*A*, *panel a*). Upon flash light illumination, a novel peak appeared at ~14 min (Fig. 3*A*, *panel b*). Based on our previous *in vitro* reconstitution experiments, we assumed that this peak might be due to Chlide *a*. To demonstrate this, another type of HPLC analysis was performed. Taking into account a recent paper of Fraser *et al.* (19), a C30 column was used. Synthetic Chlides *a* and *b* were prepared by the chlorophyllase reaction (34) and used as standards. Fig. 3*B* (*panel c*) shows that Chlide *a* and Chlide *b* were well resolved on the C30 column and also sepa-

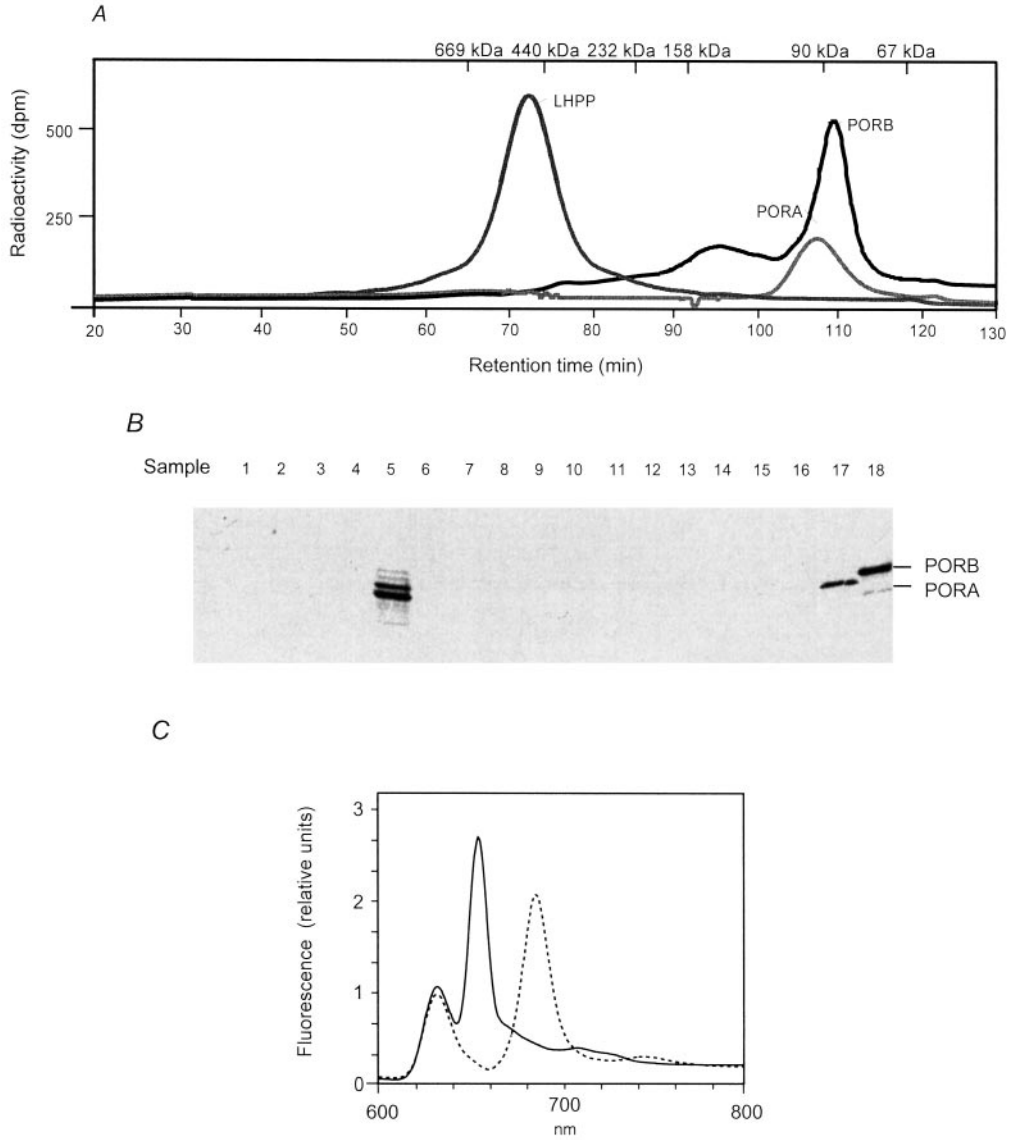

FIG. 2. **Reconstitution of a photoactive LHPP *in vitro*.** PORA-Pchlide *b*-NADPH and PORB-Pchlide *a*-NADPH ternary complexes, which had been reconstituted and purified as described in the legend to Fig. 1, were mixed and incubated in equimolar concentrations for 15 min in the dark. Then the assays were subjected to gel filtration. Individual fractions were harvested every 1 min, and aliquots were taken for radioactivity measurements in a liquid scintillation counter (*A*). Migration of marker proteins is indicated at the *top*. For tests on the presence of higher molecular weight complexes (*B*), fractions were pooled as follows: samples 1–4, fractions 1–60, containing 15 consecutive fractions each; sample 5, fractions 61–80; samples 6–16, fractions 81–104, containing two consecutive fractions each; sample 17, containing fractions 105–110; sample 18, containing fractions 112–116. In all cases, protein was recovered from the pooled fractions by precipitation with trichloroacetic acid and analyzed by SDS-PAGE. Fraction 5, containing the pooled PORA-PORB supracomplexes, was analyzed further and supplemented with a mixed galacto- and sulfolipid fraction isolated from pigment-free prolamellar bodies of barley etioplasts (*C*). The curves show low temperature spectra at −196 °C (77 K) at an excitation wavelength of 440 nm before (*solid line*) and after (*dashed line*) a single, 1-ms flash of white light. Note the quantitative conversion of Pchlide F650/657 to Chlide F684/690.

rated from Chl *a* and Chl *b*. When the pigments, eluting at ~14 min on the C18 column (see Fig. 3*A*), were applied to the C30 column, the only detectable pigment was Chlide *a* (Fig. 3*B*, *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. 4*A*), 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. 4*B*, *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 ZnPP*b* (which is chemically more inert than Pchlide *b*) and separated from nonbound pigment by gel filtra-

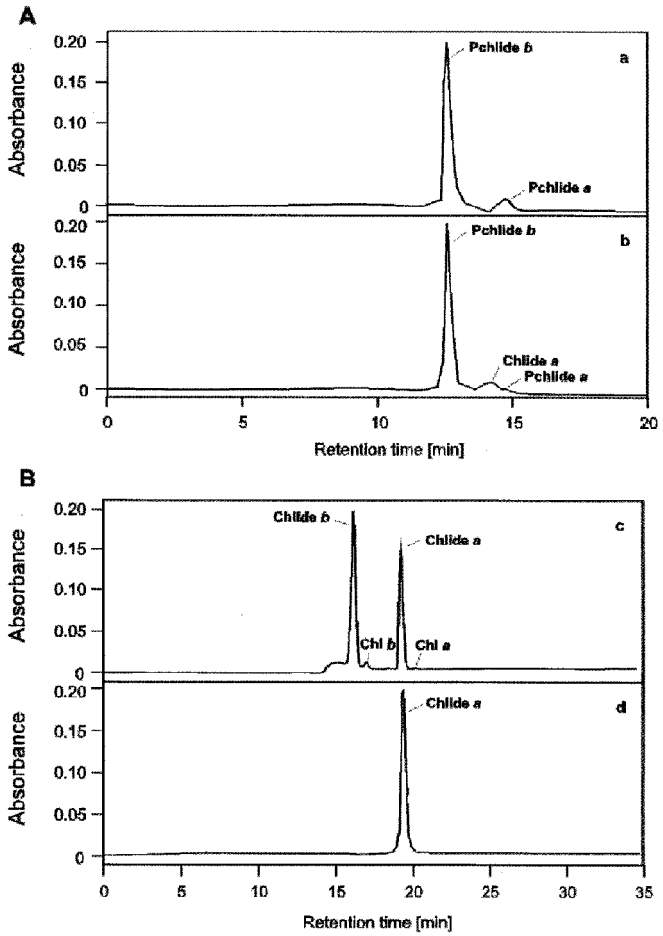

FIG. 3. **Photoconversion of Pchlide *a*, but not Pchlide *b*, in the reconstituted complex.** Higher molecular weight POR-pigment complexes were reconstituted and incubated with a mixed galacto- and sulfolipid fraction of barley prolamellar bodies as described in the legend to Fig. 2. The lipid-containing complex in turn was subjected to a single, saturating flash of white light. Pigments were extracted with 100% acetone containing 0.1% (v/v) diethyl pyrocarbonate and analyzed by HPLC. *A*, separation of natural pigments on a C18 column. Absorbance measurements were made at 455 nm, which corresponds to the Soret band of Pchlide *b*. The chromatograms show pigments before (*a*) and after (*b*) light exposure. The pigment eluting at ~12.5 min corresponds to Pchlide *b*, whereas Pchlide *a* has a retention time of ~15 min (32). *B*, identification of Chlide *a* by HPLC on a C30 column. Synthetic standards (*panel c*, Chlide *b* (16.25 min), Chl *b* (17.5 min), Chlide *a* (19.5 min), and Chl *a* (21 min)) were prepared as described under "Materials and Methods" and separated on a C30 column. Absorbance measurements were made at 435 nm. The pigment eluting at 14 min on the C18 column (*A*, *panel b*) was run in parallel (*panel d*); it corresponds to Chlide *a*.

tion, and POR-pigment complexes eluted with the flow-through exposed to white light. Parallel samples were kept in darkness. As a control, the PORB was used.

Fig. 5 shows representative room temperature fluorescence emission spectra of PORA- and PORB-bound pigments after their extraction with acetone. They revealed that the PORA indeed converted ZnPP*b* to zinc pheophorbide *b* (Fig. 5B). By analogy, also ZnPP*a* was converted to zinc pheophorbide *a* (Fig. 5*A*). Also with the PORB, the same principal results were obtained (Fig. 5, *C* and *D*, respectively). For either POR protein, a strict correlation was observed between the amounts of products formed and bound substrates, regardless of whether ZnPP*a* and *b*[2] or Pchlides *a* and *b* had been used (Fig. 5).

[2] C. Reinbothe, F. Buhr, S. Pollmann, and S. Reinbothe, unpublished data.

TABLE I

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, nondetectable pigment levels.

Pigment	Pigment (percentage of total)	
	Nonflashed	Flashed
	%	%
Pchlide *a*	22	4
Pchlide *b*	78	76
Chlide *a*	ND	20
Chlide *b*	ND	ND

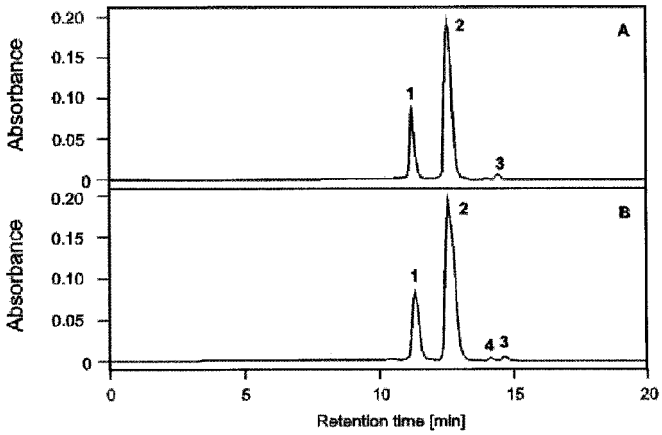

FIG. 4. **Photoconversion of Pchlide *a*, but not Pchlide *b*, in isolated prolamellar bodies.** Prolamellar bodies were isolated from barley etioplasts and either kept in darkness (*A*) or exposed to a 1-ms flash of white light (*B*). Pigments were separated by HPLC as described in Fig. 3*A* and identified 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*/*b*-POR 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

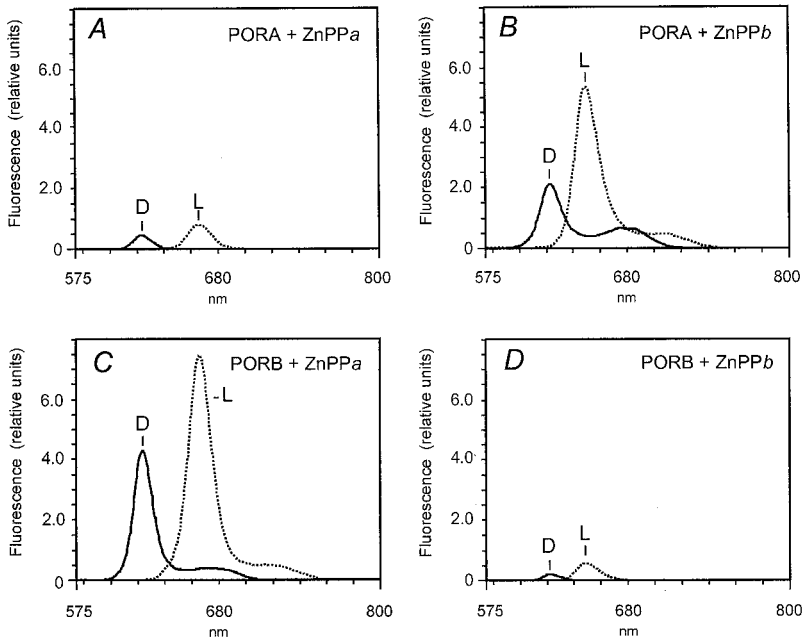

FIG. 5. **Photoconversion of synthetic ZnPP*a* and ZnPP*b* by virtue of the PORA and PORB.** *A–D*, PORA-ZnPP*a*-NADPH (*A*), PORA-ZnPP*b*-NADPH (*B*), PORB-ZnPP*a*-NADPH (*C*), and PORB-ZnPP*b*-NADPH (*D*) complexes were reconstituted *in vitro* and purified by gel filtration as described in the legend to Fig. 1. The different ternary complexes were then exposed to white light (*L*, *dotted line*) or kept in darkness (*D*, *solid line*) for 15 min. POR-substrate and POR-product complexes were extracted with acetone, and the released pigments were identified and quantified by fluorescence emission measurements at an excitation wavelength of 440 nm. The products of photoconversion, zinc pheophorbide *a* and zinc pheophorbide *b*, have emission maxima at 665 and 652 nm, respectively.

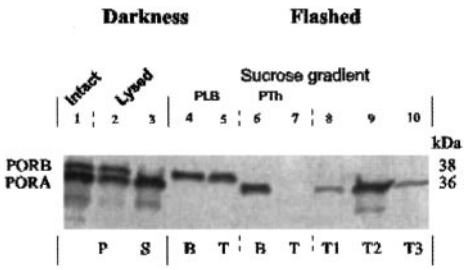

FIG. 6. **Western blot analysis of POR-related proteins.** Plastids were isolated and fractionated according to Ryberg and Sundqvist (26). The blot shows POR-related proteins in intact etioplasts (*Intact*, *lane 1*), in the pellet (*P*) and supernatant (*S*) fractions of lysed etioplasts (*Lysed*; *lanes 2* and *3*), and in the indicated prolamellar body (*PLB*) and prothylakoid (*PTh*) fractions of a top-loaded (*T*) or bottom-loaded (*B*) gradient. Note the successive solubilization of the 36-kDa PORA protein during the various steps of the procedure and that the so-called prolamellar body fraction contains only the 38-kDa PORB protein, regardless of whether the gradients were top- or bottom-loaded. Also noteworthy is that the prothylakoid fraction of the bottom-loaded gradients contains the PORA protein (*lane 6*), whereas the corresponding fraction of the top-loaded gradient is devoid of this polypeptide (*lane 7*). In this case, the PORA smears across the uppermost parts of the gradient (*lanes 8–10*, fractions T1–T3).

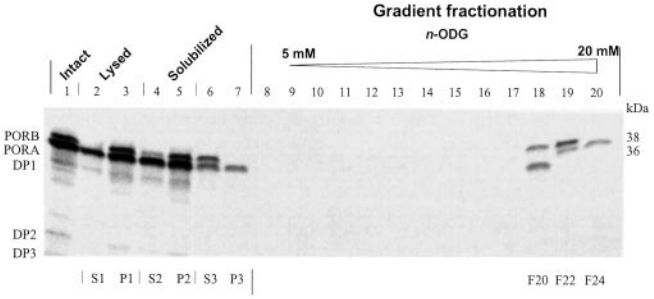

FIG. 7. **Western blot analysis of POR-related proteins during the plastid work-up procedure of Klement *et al.* (27).** The blot shows POR-related proteins in intact etioplasts (*Intact*, *lane 1*), in lysed etioplasts (*Lysed*, *lanes 2* and *3*), and in all of the different fractions obtained by stepwise detergent solubilization with *n*-octyl-β-D-glucoside (*Solubilized*, *lanes 4–7*). *P* and *S*, pellet and supernatant fraction, respectively. For DEAE-cellulose chromatography (*Gradient fractionation*), fraction S3 was used, and POR-related proteins eluted with a 5–20 mM *n*-octyl-β-D-glucoside (*n-ODG*) gradient (*lanes 9–20*). *Lane 8* shows the flow-through. Note the progressive solubilization of the bulk of the 36-kDa PORA protein during the plastid work-up procedure and that some PORA appears in the pellet fraction P3. This may be due to the high detergent concentration, favoring membrane lipid disorganization. The identity of the lower band in *lane 7* is unknown, but it may be generated by limited proteolysis of residual PORB. Noteworthy in this context is also the occurrence of several other degradation products of POR, designated *DP1–DP3*. In the ultimate POR protein preparation (pooled fractions *F20–F24*), at least three different POR proteins are found: PORA, PORB, and DP1, the pigment binding capacity of which is unknown.

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 prolamellar 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 prolamellar 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.

	Sample	
	Nonflashed	Flashed
Pigment		
Pchlide *a*	18	ND
Pchlide *b*	82	82
Chlide *a*	ND	18
Chlide *b*	ND	ND
POR		
PORA	81	81
PORB	19	19

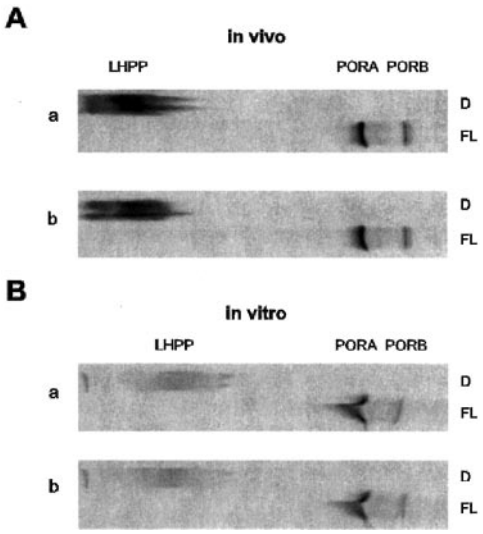

FIG. 8. **Nondenaturing, analytical PAGE of the presumed native and reconstituted lipid-containing light-harvesting complexes.** The presumed native complex was isolated from the prolamellar body of barley etioplasts (*A*) and compared with the reconstituted complex (*B*). In either case, the autoradiograms show parallel gel strips containing flashed (*FL*) and nonflashed (*D*) samples. Prior to analysis, half of the samples were illuminated with red light and immediately exposed to x-ray films at −70 °C (*panel a*), whereas the other half were blotted onto nitrocellulose and probed with the PORA antiserum (*panel b*). Higher molecular mass light-harvesting complexes indicative of LHPP are marked at the *top* of the autoradiograms. These complexes dissociate into the respective PORA-pigment-NADPH and PORB-pigment-NADPH subcomplexes upon flash light illumination.

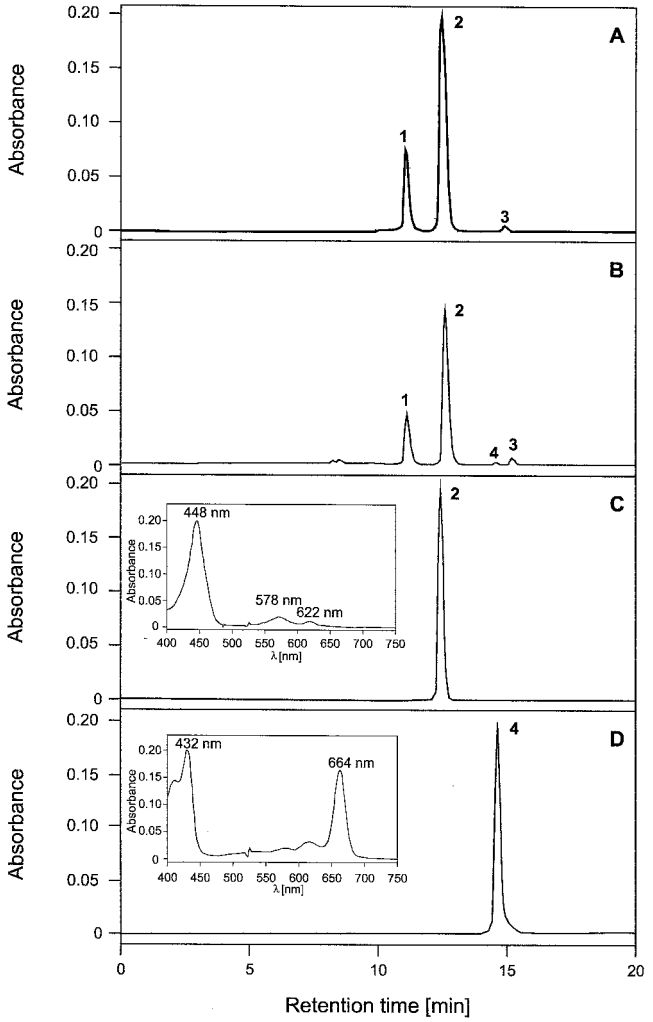

FIG. 9. **Identification of pigments contained in the electrophoretically resolved native light-harvesting complex before and after its light-induced dissociation.** Higher molecular weight POR-pigment complexes were isolated from prolamellar bodies of barley etioplasts as described in the legend to Fig. 8. The lipid-containing complexes in turn were resolved by nondenaturing PAGE. Part of the samples was subjected to a single, saturating flash of white light prior to electrophoresis (see Fig. 8), in order to induce the dissociation of the complex. After separation, individual gel slices were homogenized and extracted with acetone, and pigments were analyzed by HPLC on a C18 column. Absorbance measurements at 455 nm (*A–C*) and 435 nm (*D*) show pigments recovered from the native LHPP before (*A*) and after flash light illumination (*B*) and from the detergent-released PORA-containing (*C*) and PORB-containing (*D*) subcomplexes. The *insets* in *C* and *D* show absorption spectra of the recovered pigments.

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. 8*A* (*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. 8*A*, *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. 8*A*, *FL*). Upon scaling up the procedure 1000-fold, these could be identified as PORA and PORB by protein sequencing (data not shown). Their approximate stoi-

chiometry 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 nondenaturing PAGE, a similar, although slightly smaller, complex could be seen (Fig. 8*B*). 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. 9*A*, *peak 2* and *3*, respectively). In addition, substantial amounts of 7-hydroxy-Pchlide *a* could also be seen (Fig. 9*A*, *peak 1*). Upon flash light illumination, correlating with the disintegration of the complex (see Fig. 8*A*), only Chlide *a* was produced (Fig. 9*B*, *peak 4*). It co-migrated with the PORB protein band (Fig. 9*D*). Protochlorophyllide *b*, by contrast, remained quantitatively unchanged (Fig. 9, compare *B versus A*) and co-migrated with the PORA protein band (Fig. 9*C*). 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 nonphotoconvertible.

DISCUSSION

In the present study, we addressed three different questions. First, would the PORA and PORB display the same stringent substrate specificities for their presumed natural substrates (Pchlide *b* and Pchlide *a*, respectively) as reported previously for their zinc counterparts (5)? 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 PORA binds ~10-fold higher amounts of Pchlide *b* (ZnPP*b*) relative to Pchlide *a* (ZnPP*a*). PORB, by contrast, was specific for Pchlide *a* (ZnPP*a*) and bound ~10-fold lower levels of Pchlide *b* (ZnPP*b*). 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 conclude 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 barley 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 (35–41). Tanaka *et al.* (36) cloned a *Chlamydomonas reinhardtii* cDNA for a putative Chlide *a* oxygenase. Later studies by Espineda *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 Pchl(ide) *b* and Chl(ide) *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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