Identification of plastid envelope proteins required for import of protochlorophyllide oxidoreductase A into the chloroplast of barley

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Chloroplasts synthesize an abundance of different tetrapyrrole compounds. Among them are chlorophyll and its precursor protochlorophyllide (Pchlide), which accumulate in light- and dark-grown plants, respectively. Pchlide is converted to chlorophyllide by virtue of the NADPH:Pchlide oxidoreductase (POR), which, in angiosperms, is the only known light-dependent enzyme of the chlorophyll biosynthetic pathway. In etiolated barley plants, two closely related POR proteins exist termed PORA and PORB, which are nuclear gene products. Here we identified plastid envelope proteins that interact with the cytosolic PORA precursor (pPORA) during its posttranslational chloroplast import. We demonstrate that pPORA interacts with several previously unreported components. Among them is a Pchlide *a* oxygenase, which provides Pchlide *b* as import substrate for pPORA, and a tyrosine aminotransferase thought to be involved in the synthesis of photoprotective vitamin E. Two other constituents were found to be orthologs of the GTP-binding proteins Toc33/34 and of the outer plastid envelope protein Oep16.

Tetrapyrroles, such as heme, chlorophyll, and phytochromobilin, play important roles in higher plants. They are involved in various processes such as light absorption and energy transduction during photosynthesis, photoreception by phytochrome, and the control of nuclear gene expression (1–3). All the different tetrapyrroles are formed by virtue of the same C5 pathway (4). Previous work has shown that part of the C5 pathway is localized in the plastid envelope (summarized in ref. 5).

Only few examples exist of proteins that may be imported into the plastid in a tetrapyrrole-regulated manner. Eggink *et al.* (6) proposed that import and membrane insertion of the nucleus-encoded chlorophyll-binding proteins may be regulated by "retention signals" in the mature polypeptides that anchor the proteins to the inner plastid envelope membranes. Only in the presence of nascent chlorophyll *b* would import occur (6). We demonstrated that the nucleus-encoded NADPH:protochlorophyllide (Pchlide) oxidoreductase A (PORA) is imported into the plastids in a substrate-dependent manner (7–9). The transit peptide of cytosolic precursor PORA (pPORA) contains a porphyrin-binding site, which is likely to bind Pchlide during import (10). Several lines of evidence (summarized in ref. 11) suggest that pPORA does not interact with the previously identified translocons of the outer (Toc) and inner (Tic) chloroplast envelope (for review, see refs. 12–14).

The components of the Toc complex have been studied in detail and were shown to consist of the main precursor protein receptor Toc159, the translocation channel protein Toc75, and the GTP-binding protein Toc34 (15–18). Recent work provoked the view that several versions of the Toc complex may exist in *Arabidopsis*, which could differ by an interchange of receptor components. Bauer *et al.* (15) identified two Toc proteins in addition to the previously observed main preprotein receptor protein Toc159 (16, 17). Jarvis *et al.* (18) demonstrated that "twin components" of the same regulatory GTP-binding protein, Toc34 and Toc33, are differentially expressed during plant development. In 1991, Dahlin and Cline (19) observed that protein import is determined by developmental fate and age of the plant, implying a rather flexible nature of the responsible import machinery.

In the present work, plastid envelope proteins were identified that interact with pPORA during its posttranslational import into the chloroplast. By using a carboxyl-terminally hexahistidine-tagged precursor and chemical crosslinking, we demonstrate that pPORA interacts with several unique plastid envelope membrane proteins that form a Pchlide-containing protein import site.

Materials and Methods

DNA Constructs. cDNA clones A7 and L2, encoding the pPORA and pPORB (20), respectively, were subcloned into the pQE16 vector (Qiagen, Hilden, Germany) according to the manufacture's instructions. The resulting clones encode proteins that contain hexahistidine tags at their carboxyl termini. The identity of the different constructs was confirmed by DNA sequencing with a T7 DNA sequencing kit and the gel system described in ref. 21 or by GATC Biotech (Constance, Germany).

Plastid Isolation, Manipulation, and Protein Import. Seeds of barley (*Hordeum vulgare* cv. Carina) were germinated at 25°C and grown either in complete darkness or under continuous white-light illumination provided by fluorescent bulbs (30 W/m^2) for 5 days. For the experiment described in Fig. 7 (which is published as supporting information on the PNAS web site), barley plants were grown in alternating 10-h light and 14-h dark cycles (7) for an identical period. Plastids were isolated from homogenized leaf tissues by density gradient centrifugation on Percoll (Pharmacia LKB) (7). Energy depletion was achieved as described by Theg *et al.* (22). Treatment of isolated chloroplasts with phosphate-buffered 5-aminolevulinic acid was performed as described (7). Controls were mock-incubated with phosphate buffer instead of 5-aminolevulinic acid.

Reisolated, intact plastids were resuspended in import buffer lacking ATP (7) and added to the radiolabeled precursors given in the text. Either bacterially expressed precursors (see below) or *in vitro* synthesized precursors were used. *In vitro* synthesis of ^{35}S-containing precursors was made in a wheat germ system by coupled transcription/translation of respective cDNA clones (20). Precursors were concentrated by ammonium sulfate precipitation. All precursors were denatured in 8 M urea before use (11). To study import, aliquots were withdrawn and diluted such that the final urea concentration in the assays did not exceed 0.2 M. Final 50-μl import

Abbreviations: Pchlide, protochlorophyllide; POR, NADPH:Pchlide oxidoreductase; pPORA, precursor POR A; Toc, translocon of the outer chloroplast envelope; Tic, translocon of the inner chloroplast envelope; Ptc, Pchlide-dependent translocon; NTA, nitrilotriacetic acid; APDP, ^{125}I-*N*-[4[(*p*-azidosalicylamido)butyl]-3′(2-pyridyl-dithio)propionamid; OM, outer-membrane; OM-IM, intermediate-density membranes; IM, inner-membrane.

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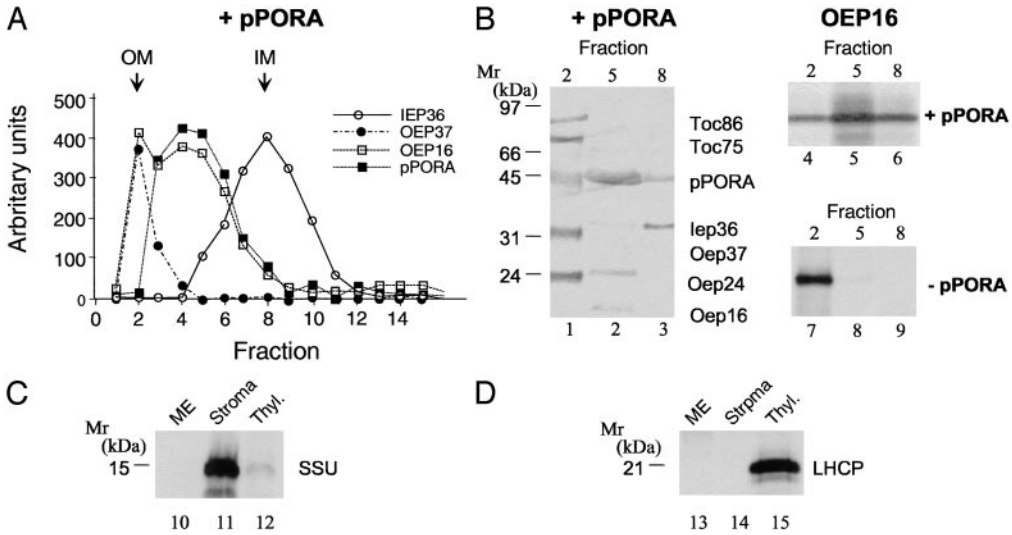

Fig. 1. Generation of the ^{35}S-pPORA-(His)$_6$ import intermediate. Isolated barley chloroplasts were incubated with bacterially expressed, urea-denatured ^{35}S-pPORA-(His)$_6$ in the presence of 0.1 mM Mg-ATP and 0.1 mM Mg-GTP. After 15 min in darkness, the plastids were diluted into ice-cold import buffer lacking ATP and GTP and reisolated. The chloroplasts were then lysed under hypertonic conditions, and total envelope membranes were subfractionated by flotation into linear sucrose gradients (20–38%). (*A*) Distribution of Oep37, Iep36, Oep16, and the ^{35}S-pPORA-(His)$_6$ in outer membranes (OM, fractions 1 and 2), inner membranes (IM, fractions 8–10), and OM-IM junction complexes (fractions 3–7) obtained after subfractionation. (*B*) Same as in *A*, but showing, in addition, distribution of Toc86, Toc75, Oep24 (lanes 1–3), and Oep16 (lanes 4–6) in gradient fractions 2 (lanes 1 and 4), 5 (lanes 2 and 5), and 8 (lanes 3 and 6), respectively (+pPORA). Lanes 7–9 show the distribution of Oep16 in a gradient of crude envelopes of chloroplasts that had been incubated in the absence of ^{35}S-pPORA-(His)$_6$. Each lane contained 10 μg of protein. Note that the larger blot (lanes 1–3) showing the different outer- and inner-envelope proteins was probed with a heterologous antiserum against Oep16 of pea, whereas the two smaller blots were developed with a homologous antiserum against barley Oep16. (*C* and *D*) Immunoblot of ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (SSU) and light-harvesting chlorophyll *a*/*b*-binding protein of photosystem II (LHCP) with protein from crude envelope membranes (ME, lanes 10 and 13) before their separation on sucrose gradients, and stromal (Stroma, lanes 11 and 14) and thylakoid (Thyl., lanes 12 and 15) fractions.

mixtures usually consisted of 25 μl of doubly concentrated import buffer (7), 10 μl of the plastid suspension containing, if not stated otherwise, $5{\cdot}10^7$ plastids, 5 μl of the different urea-denatured, radiolabeled precursors, and Mg-ATP and Mg-GTP as indicated in the figure legends. If needed, double-distilled water was added to adjust the final reaction volume. All assay mixtures were assembled on ice under a dim green safe light; the actual import reactions were performed at 23°C for 15 min in darkness. Plastids were reisolated on Percoll after import (7). Postimport protease treatment of plastids with thermolysin and extraction of membranes with sodium carbonate (pH 11) or 1 M NaCl were made as described (23). Plastid subfractionation into envelopes, stroma, and thylakoids was performed according to Li *et al.* (24). Protein was precipitated with trichloroacetic acid [5% (wt/vol) final concentration], resolved by SDS/PAGE on 10–20% (wt/vol) polyacrylamide gradients (25) either under reducing or nonreducing conditions (26), and detected by autoradiography.

Production of Import Intermediates. ^{35}S-Precursors were expressed in *Escherichia coli* strain SG13009 (Qiagen) and recovered from inclusion bodies by dissolution in 8 M urea containing 20 mM imidazole-HCl (pH 8.0). Protein was passed over a G-25 column, which yielded ≈85% pure precursor fractions. This ^{35}S-pPORA-(His)$_6$ was, in turn, incubated with isolated, energy-depleted barley chloroplasts in the presence of 0.1 mM Mg-ATP and 0.1 mM Mg-GTP for 15 min in darkness. After the reaction, the plastids were reisolated and lysed under hypertonic conditions; total membranes were subfractionated by flotation into linear 20–38% sucrose gradients (27). Individual fractions were retrieved from the gradients, and protein was precipitated with trichloroacetic acid, separated by SDS/PAGE, and transferred onto nitrocelluose filters. The filters were then subjected either to autoradiography, to detect ^{35}S-pPORA-(His)$_6$, or to immunoblotting with antisera against Toc86, Toc75, Oep24, Oep16, and Iep36. As a control, import and plastid fractionation were performed in the absence of ^{35}S-pPORA-(His)$_6$. For the experiment described in Fig. 4, individual gradient fractions were extracted with 100% acetone and centrifuged, and the pigments contained in the supernatant were analyzed by HPLC and subsequent absorbance and fluorescence measurements (see below), whereas protein recovered in the respective sediments was analyzed by SDS/PAGE and Western blotting with antisera against Ptc52 and Oep16.

Protein import complexes were solubilized from the intermediate-density fraction in a buffer containing 2% Triton X-100, 50 mM Tris·HCl (pH 7.5), 300 mM NaCl, 20 mM imidazole-HCl (pH 8.0), and 1 mM PMSF (26, 27). After a step of centrifugation at 100,000 × *g* for 15 min, 10-ml portions of the supernatant were incubated for 1 h at 4°C with 0.25 ml Ni^{2+}-nitrilotriacetic acid (NTA)-agarose beads in solubilization buffer (26). The beads were washed twice, and the bound protein was eluted with 2% SDS/100 mM EDTA/50 mM Pipes-NaOH (pH 7.4), precipitated by methanol/chloroform, suspended in SDS sample buffer (25), and loaded on a SDS/10–20% polyacrylamide gel. The gel was stained with Coomassie blue and subjected to autoradiography. Individual bands were excised from replicate gels and subjected to 2D SDS/PAGE, including isoelectric focusing in the first dimension and SDS/PAGE in the second dimension (28). Single spots from 20 such gels were pooled and subjected to protein sequencing.

Crosslinking. Derivatization of precursors with ^{125}I-*N*-[4[(*p*-azidosalicylamido)butyl]-3′(2-pyridyl-dithio)propionamid (APDP) and subsequent crosslinking were carried out as described in ref. 29.

Pigment Measurement. Pigment measurements were made by HPLC on a C18 reverse-phase silica gel column (Hypersil ODS, 5 μm, Shandon, Pittsburgh), with synthetic Pchlides *a* and *b* as standards (30).

Immunological Techniques. Antisera against Toc86, Toc75, Oep37, Oep16, Oep24, and Iep36 have been described (27, 29, 31, 32). The

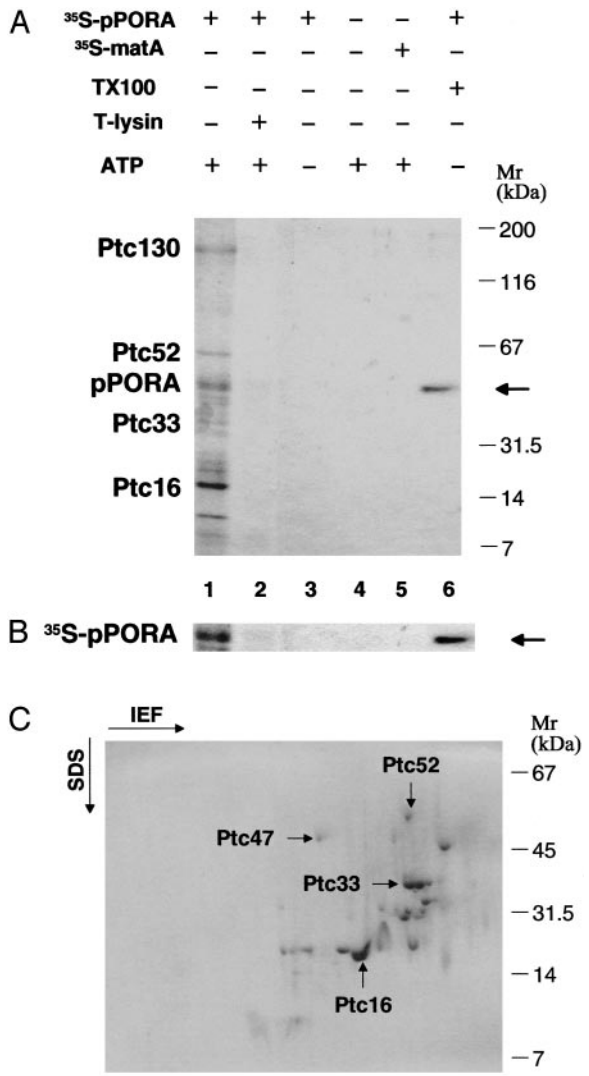

Fig. 2. Purification on Ni^{2+}-NTA-agarose of envelope proteins bound to ^{35}S-pPORA-$(His)_6$. Import intermediates were prepared as described in Fig. 1 and solubilized from reisolated envelope membranes with 2% Triton X-100 (TX100). ^{35}S-pPORA-$(His)_6$-containing protein complexes were then purified on Ni^{2+}-NTA-agarose and analyzed by SDS/PAGE. (*A*) Coomassie stain of recovered envelope proteins. The positions of marker proteins are indicated. Ptc stands for Pchlide-dependent translocon protein; the number denotes the relative molecular mass. (*B*) Autoradiogram of ^{35}S-pPORA-$(His)_6$ (arrow) in gradient fractions that should contain OM-IM complexes. (*C*) Coomassie stain of two-dimensionally separated Ptc proteins. Protein equivalent to 150 μg of BSA was resolved by isoelectric focusing (IEF) in the first dimension (left to right, pH 5–9) and SDS/PAGE (SDS, top to bottom) in the second dimension.

antiserum against Ptc52 was raised against the synthetic peptide ^{165}CVYHGWCFDGRGSCQF^{180} (the numbering refers to dbEST clone BF366467) which corresponds to the second determined amino acid sequence motif of the protein (see Fig. 3). The anti-Tam1 antiserum used to detect Ptc47 is described in ref. 33. Immunoprecipitation was performed according to ref. 34 with the antisera described in the text. Western blotting was done as described by Towbin *et al.* (35) by using the anti-rabbit, anti-goat, alkaline phosphatase system, and the indicated antisera.

Results and Discussion

Isolation of Components of the Pchlide-Dependent Translocon (Ptc) Complex. Plastid envelope proteins that interact with pPORA were identified. The precursor was expressed as a C-terminal hexahistidine-tagged protein in *E. coli* and purified (26) and incubated with isolated, energy-depleted (22) barley chloroplasts in the presence of 0.1 mM Mg-ATP and 0.1 mM Mg-GTP. Pilot experiments revealed that these nucleoside triphosphate concentrations yielded highest amounts of envelope-bound pPORA (Fig. 7). After incubation, the plastids were reisolated, rapidly disrupted, and processed to yield a crude envelope fraction (27). This fraction was loaded onto a sucrose gradient and separated by flotation to obtain a light outer-membrane (OM) fraction, an intermediate-density membrane (OM-IM) fraction, and a slightly denser inner-membrane (IM) fraction (27) (Fig. 1*A*). Immunoblots showed that most of the outer-envelope membrane marker proteins Toc86, Toc75, Oep37, and Oep24 were present in the light OM fraction, and most of the inner-envelope membrane protein Iep36 was present in the IM fraction (Fig. 1*B*, +pPORA). The OM-IM fraction contained low amounts of Oep37 and Iep36 (Fig. 1 *A* and *B*) and therefore consisted of fragments of outer and inner membranes, presumably held together by contact sites (27). A large proportion of pPORA-$(His)_6$ was found in the OM-IM fraction, but some precursor was also present in the OM and IM fractions (Fig. 1 *A* and *B*). The previously identified 16-kDa protein, which chemically crosslinks to pPORA (36), was likewise present in all three fractions and copurified in approximately stoichiometric amounts with the pPORA, as estimated from the staining intensities on the blots in Fig. 1. When isolated chloroplasts were incubated in the absence of pPORA-$(His)_6$ and fractionated as described, the 16-kDa protein was largely restricted to the OM fraction (Fig. 1*B*). This control demonstrated that OM-IM junction complexes were increased in the presence of precursor. Immunoblot analyses of stripped filters with homologous antisera confirmed that OM, OM-IM, and IM fractions obtained in the presence of pPORA-$(His)_6$ were not significantly contaminated with stromal and thylakoid proteins, such as the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase and the major light-harvesting chlorophyll *a*/*b*-binding protein of photosystem 2 (data not shown). Either protein strongly reacted in isolated stromal and thylakoid protein fractions, however (Fig. 1 *C* and *D*).

The OM-IM fraction was subjected to detergent solubilization (27) to recover envelope proteins bound to pPORA-$(His)_6$. The resulting higher molecular weight complexes were purified by Ni^{2+}-NTA-agarose (26) and analyzed by SDS/PAGE, Coomassie staining (Fig. 2*A*), and autoradiography (Fig. 2*B*). The eluted

Conserved Rieske center binding site

LLS1	A.th.	^{130}DLCPHRLAPLSEGRLDENGHLQCSYHGWSFGGCGS^{164}
LLS1	Z.m.	^{113}DRCPHRLAPLSEGRIDETGCLQCSYHGWSFDGCGS^{147}
TIC55	P.s.	^{142}DRCPHRLAKLSEG-QLIDGRLECLYHGWQFEGEGK^{175}
TIC55	A.th.	^{127}DRCPHRLAKLSEG-QLIDGRLECLYHGWQFEGEGK^{161}
CAO	A.th.	^{260}NTCAHR-CPLDLGTVNE-GRIQCPYHGWEYSTDGE^{292}
PTC52	H.v.	^{143}DACPHRLAPLSEGRIDDKGRLQCVYHGWCFDGRGS^{177}

Conserved mononuclear iron binding site

LLS1	A.th.	^{246}LMENVSDPSHIDFAHAK^{262}
LLS1	Z.m.	^{228}LMENVSDPSHIEFAHHK^{244}
TIC55	P.s.	^{248}LLENLMDPAHVPISHDR^{264}
TIC55	A.th.	^{233}LLENLMDPAHVPISHDR^{249}
CAO	A.th.	^{358}LLDNLLDLAHAPFTHTS^{374}
PTC52	H.v.	^{486}LVENLMDPAHGPLSHHK^{543}

Fig. 3. Identification of Ptc52 as a member of the Lls1, Tic55, and Cao superfamily. Ptc52 was purified and sequenced. Three partial amino acid sequences were obtained which matched with the conserved Rieske center-binding and mononuclear iron-binding motifs of Lls1 of maize (AAC49676) and *Arabidopsis* (AAC49679), Tic55 of pea (CAA04157) and *Arabidopsis* (AAD23030), and Cao of *Arabidopsis* (BAA82484). A protein likely to represent Ptc52 was deduced from EST BF266467 that covers both sequence domains. *Staphylococcus aureus* V8 protease- and endoproteinase Lys C-derived peptide sequences are overlined and underlined, respectively.

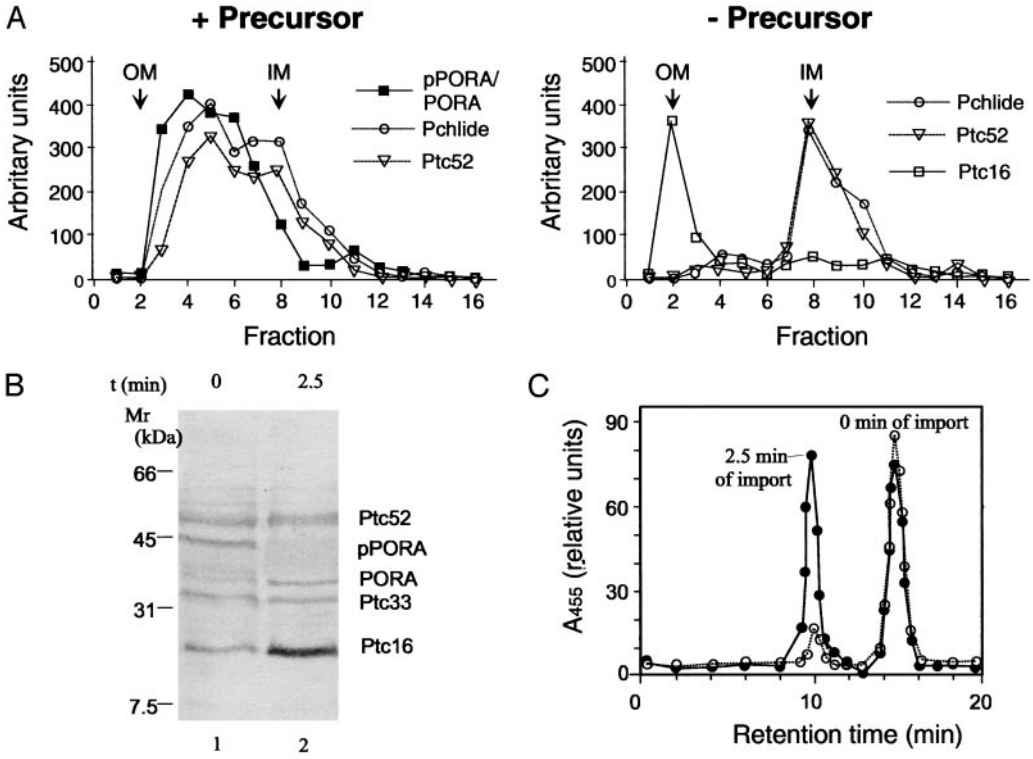

Fig. 4. Detection of Ptc52 and Pchlide *b* in OM-IM junction complexes. (*A*) An antiserum was raised against a fingerprint amino acid sequence of Ptc52 (see *Materials and Methods*). This antiserum was used to determine the distribution of Ptc52 in outer membranes (OM, fractions 1 and 2), inner membranes (IM, fractions 8–10), and OM-IM junction complexes (fractions 3–7). As a control, membrane fractions of chloroplasts that had not been incubated with ^{35}S-pPORA-(His)$_6$ (−precursor) were probed identically. Pchlide levels determined in parallel are indicated. (*B*) Import and processing of the envelope-bound ^{35}S-pPORA-(His)$_6$. ^{35}S-pPORA-(His)$_6$ was bound to chloroplasts in the presence of 0.1 mM Mg-ATP and 0.1 mM Mg-GTP, and the plastids were reisolated. The plastid-bound precursor then was chased into the chloroplasts by raising the Mg-ATP concentration to 2 mM and supplementing the assay mixtures with 5-aminolevulinic acid (0.5 mM final concentration). At time zero (0) and 2.5 min after the beginning of the incubation, protein bound to the precursor was recovered from OM-IM junction complexes containing ^{35}S-pPORA-(His)$_6$ by Triton X-100 solubilization, as described in Fig. 2, and analyzed by SDS/PAGE and Coomassie staining. The positions of the envelope-bound pPORA and processed PORA are indicated. The copurifying 52-, 33-, and 16-kDa proteins are marked. (*C*) HPLC analysis of pigments recovered after 0 and 2.5 min of import. Pigments were extracted with 100% acetone containing 0.1% (vol/vol) diethylpyrocarbonate from the import reactions described in *B* and separated by HPLC on a C18 column.

protein consisted of the pPORA-(His)$_6$; four main polypeptide bands of 130, 52, 33, and 16 kDa; and a few other, less abundant bands (Fig. 2*A*, lane 1). The four main bands copurified with the pPORA-(His)$_6$ in approximately stoichiometric amounts in the 1D separation, as judged from the staining intensities in Fig. 2*A* (lane 1).

Several control experiments were performed to demonstrate that the four copurifying polypeptide bands interacted with pPORA specifically. Fig. 2*A* demonstrates that neither pPORA-(His)$_6$ nor copurifying envelope proteins were detected when the chloroplasts were treated with thermolysin before import (lane 2). As shown in ref. 7, pretreatment with thermolysin abolishes the capability of the chloroplast to import pPORA. Second, omission of Mg-ATP in the import reaction yielded neither the pPORA-(His)$_6$ nor the copurifying polypeptides bands (Fig. 2*A*, lane 3), a result consistent with the published requirement of pPORA import for Mg-ATP (7). Third, an import reaction in the absence of import substrate did not yield envelope polypeptides that bound to Ni^{2+}-NTA-agarose (Fig. 2*A*, lane 4). This control excluded the possibility that envelope polypeptides present in residual OM-IM junction complexes formed in the absence of precursor bound nonspecifically to the affinity column. Fourth, the use of a mature PORA-(His)$_6$ protein, which lacked the transit peptide of pPORA, as import substrate yielded neither the radiolabeled protein nor the copurifying polypeptides (Fig. 2*A*, lane 5), as would be expected from the published dependence of import on the transit sequence (10). Finally, no envelope polypeptides copurified with import substrate when it was added during the solubilization of the OM-IM envelope fraction (Fig. 2*A*, lane 6). This finding excluded the possibility that pPORA-(His)$_6$ interacted with envelope polypeptides in an import-independent reaction. Fig. 2*C* shows a representative 2D separation of the envelope polypeptides that specifically copurified with pPORA-(His)$_6$. These envelope proteins are referred to as Ptcs and are denoted by a number that indicates their molecular mass throughout the rest of the article. In several cases, groups of Ptc proteins with identical molecular mass values but different pI values copurified with pPORA-(His)$_6$. Some Ptc proteins and the pPORA-(His)$_6$ were not resolved on the gel, presumably because of their strongly basic or acidic isoelectric points. The indicated four protein spots, Ptc52, Ptc47, Ptc33, and Ptc16, were sequenced, and the obtained amino acid sequences were aligned with those available in the data banks.

Oep16 and a Toc33/34-Like Protein Are Part of the Ptc Complex. The first identified protein, Ptc16, corresponds to a previously identified outer-envelope protein of barley (31) and pea (32) chloroplasts referred to as Oep16. This protein was associated with the OM-IM complex (Fig. 1) and also had been identified as a crosslink partner of pPORA and transit peptide of pPORA-dihydrofolate reductase, a derivative in which the transit peptide of pPORA had been fused to a cytosolic dihydrofolate reductase reporter protein of mouse (36).

The second identified protein, Ptc33, is closely related to both Toc34 and Toc33 of *Arabidopsis* (18) (see Fig. 8, which is published as supporting information on the PNAS web site). Only expressed sequence tags coding for a Toc34-like protein were found in the

data banks for barley, which at first glance could suggest that Ptc33 is an ortholog of Toc34 (Fig. 8). However, the partial amino acid sequence obtained spanned a region in which Toc34 and Toc33 of *Arabidopsis* are highly similar (18), not permitting a definitive answer as to the nature of Ptc33 at this stage.

The Ptc Complex Contains Pchlide and a Putative Pchlide *a* Oxygenase. The third identified component, Ptc52, displays similarity to three, at first glance, quite unrelated proteins: the lethal leaf spot protein Lls1 (37), Tic55 (38), and the recently identified chlorophyllide *a* oxygenase (Cao) (19) (Fig. 3). Whereas Lls1 was proposed to operate as a suppressor of cell death in plants (37), Tic55 has been identified as part of the Tic complex (38). Cao, by contrast, is implicated in chlorophyllide *b* synthesis (40, 41). All three proteins, Lls1, Tic55, and Cao, have in common a consensus sequence (Cys-X-His-X_{16-17}-Cys-X_2-His) for coordinating a Rieske-type [2Fe–2S] cluster (42) and a conserved mononuclear non-heme Fe-binding site (Glu-X_{3-4}-Asp-X_2-His-X_{4-5}-His; ref. 43) (Fig. 3). The determined amino acid sequences of barley Ptc52 fall into these two highly conserved regions (see Fig. 3).

The similarity of Ptc52 to Cao prompted us to conclude that the protein may be involved in Pchlide *a*-to-Pchlide *b* conversion and thus may play a key role for the regulation of pPORA import. To test this hypothesis, OM, OM-IM, and IM fractions which had been prepared as described in Fig. 1, were extracted with acetone and traced for the presence of Pchlide by HPLC and Ptc52 by Western blotting. Fig. 4*A* demonstrates that, in the presence of precursor, maximum amounts of both Pchlide and Ptc52 were found in the OM-IM fraction. In the absence of added precursor either constituent was present almost exclusively in the IM fraction, however (Fig. 4*A*, −precursor). When we performed a chase experiment in which the envelope-bound pPORA was allowed to translocate into the stroma in the presence of 0.5 mM 5-aminolevulinic acid plus 2 mM Mg-ATP and 0.1 mM Mg-GTP (Fig. 4*B*), increasing amounts of Pchlide *b* copurified with the recovered import complex, as compared with the import complex obtained at time zero (Fig. 4*C*, compare 2.5- and 0-min import). Consistent with previous results (30), Pchlide *b* (peak 1) has a retention time of 9.5 min, and Pchlide *a* (peak 2) has a retention time of 15 min. Photodiode array detector-based absorbance measurements made during HPLC confirmed the identity of the two compounds.

A Tyrosine Aminotransferase Implicated in α-Tocopherol Biosynthesis Is a Ptc Protein. The fourth identified Ptc protein, Ptc47, displayed amino acid sequence similarity to a recently identified tyrosine aminotransferase of *Arabidopsis* (33). In plants and bacteria, these enzymes catalyze the transfer of an amino group from tyrosine to 2-oxoglutarate, giving rise to 4-hydroxyphenylpyruvate. This compound is converted to either plastoquinone or α-tocopherol (vitamin E) by homogentisate. In *Arabidopsis*, a small protein family exists, comprising six different members, of which the cDNA-encoded Tam1 protein (renamed from clone AtTat1, CAA23025) displays tyrosine aminotransferase activity *in vitro* (33).

A heterologous antiserum (33) was used to study the localization of Ptc47 and its association with the Ptc complex. As shown in Fig. 5*A*, the antiserum reacted with a 47-kDa band in OM-IM junction complexes containing the pPORA-$(His)_6$ (lane 2), but it was detectable in the IM fraction in the absence of added pPORA-$(His)_6$ (lane 6). A 47-kDa polypeptide was precipitated among the protein of detergent-solubilized OM-IM junction complexes containing the pPORA-$(His)_6$ and Ptcs (Fig. 5*B*, lane 10). In thylakoid and stromal fractions or a whole-leaf soluble fraction of barley plants, no crossreactive 47-kDa protein band was detectable (Fig. 5*B*, lanes 7–9), whereas total chloroplasts contained such band (Fig. 5*C*, lane 11). Protease treatment of total chloroplasts with thermolysin and trypsin confirmed the IM localization of the 47-kDa protein, because it was only sensitive to trypsin treatment (Fig. 5*C*, lanes 12 and 13). Trypsin is known to attack both outer- and inner-envelope membrane proteins and to break down IM proteins up to their membrane-anchoring domains (44). Consistent with this view, the 47-kDa band was converted into a smaller, ≈15 kDa degradation product (Fig. 5*C*, lane 13). Resistance of isolated inner-envelope membranes against extraction with 1 M NaCl or 0.1 M Na_2CO_3 (pH 11; ref. 44) (Fig. 5*C*, lanes 14–17) proved that the 47-kDa protein is an integral membrane protein. Coimmunoprecipitations of detergent-solubilized OM-IM junction complexes containing the Ptcs showed that Ptc47 is likely to interact with Ptc52 (Fig. 5*D*).

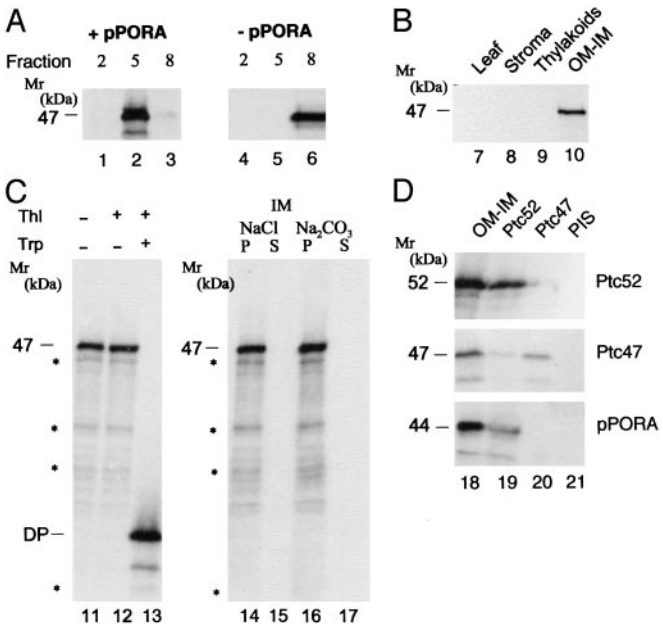

Fig. 5. Ptc47 is an inner plastid envelope membrane protein which interacts with Ptc52. (*A*) Import intermediates were prepared in the presence of ^{35}S-pPORA-$(His)_6$ (+pPORA, lanes 1–3), and total membranes were fractionated on sucrose gradients as described in Fig. 1. As a control, total membranes were prepared from chloroplasts that had not been incubated with ^{35}S-pPORA-$(His)_6$ (−pPORA, lanes 4–6). The Western blot shows the distribution of Ptc47 in the indicated gradient fractions, which correspond to those shown in Fig. 2. (*B*) Chloroplasts were isolated from light-grown barley plants and fractionated into stroma (lane 8) and thylakoids (lane 9). As a control, OM-IM junction complexes containing pPORA-$(His)_6$ were prepared and solubilized as described in Figs. 1 and 2 (OM-IM, lane 10). For comparison, a total leaf extract was used (lane 7). Protein (10 μg) found in each of these different fractions was resolved by SDS/PAGE and blotted, and the filters were probed with a heterologous antiserum against a tyrosine aminotransferase of *Arabidopsis*. (*C*) Isolated chloroplasts (lanes 11–13) or inner-envelope membranes (lanes 14–17) prepared from isolated intact barley chloroplasts were treated with thermolysin (Thl) and trypsin (Trp) as indicated (lanes 11–13) or extracted with 1 M NaCl or 0.1 M Na_2CO_3 (pH 11, lanes 14–17). Each assay was centrifuged, and protein found in the resulting pellet (P, lanes 14 and 16) and supernatant (S, lanes 15 and 17) were fractions detected by Western blotting as described. In lane 13, 50 times the amount of protein loaded in lanes 11 and 12 was used. (*D*) Coimmunoprecipitation of proteins solubilized from OM-IM junction complexes containing pPORA-$(His)_6$ (see Fig. 2) with the indicated anti-Ptc52 and anti-Ptc47 antisera and respective preimmune sera (PIS). After SDS/PAGE, the blots of the immunoprecipitates were developed with the anti-Ptc52, anti-Ptc47, and anti-pPORA antisera marked to the right.

Photo-Crosslinking of Ptc Proteins. Photo-crosslinking was used to verify the interaction of pPORA with the different Ptc proteins. pPORA was derivatized at its cysteine residues to bear APDP, which is a hetero-bifunctional, photoactivatable, cleavable crosslinker (29). Modification of pPORA with APDP did not affect the overall properties of the precursor, as deduced from the *in vitro* processing experiments shown in Fig. 6*A*. APDP-activated pPORA was incubated with isolated, energy-depleted barley chloroplasts in

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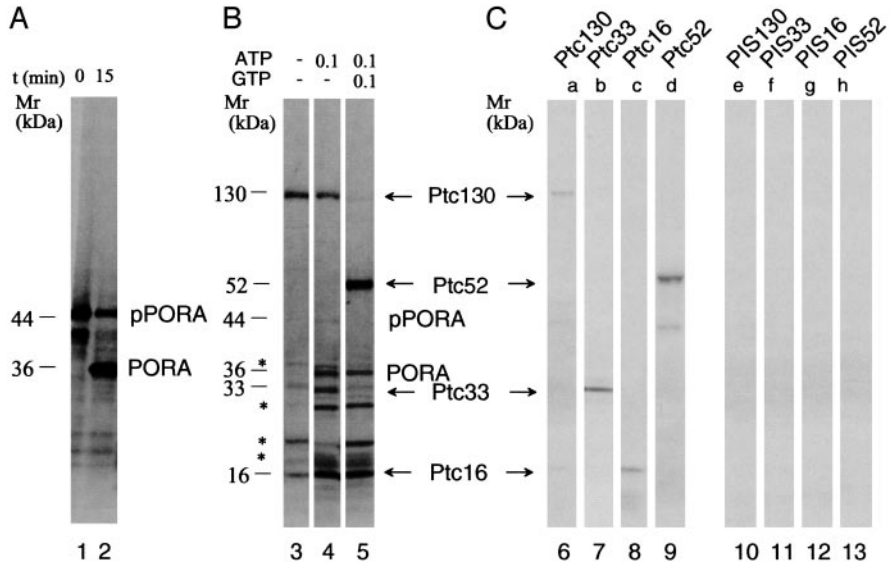

Fig. 6. Label transfer crosslinking of envelope polypeptides at different stages of import. (*A*) Detection of APDP-pPORA (lane 1) and its mature form (lane 2) generated in an *in vitro* processing experiment. (*B*) Crosslinking of envelope polypeptides with APDP. Energy-depleted barley chloroplasts were incubated at 23°C in the dark with APDP-pPORA in the presence of Mg-GTP and Mg-ATP (in millinolar concentrations). After 15 min, the samples were irradiated at 312 nm for 20 min on ice. Chloroplasts were reisolated and fractionated, and total envelope protein was solubilized with SDS and analyzed by reducing SDS/PAGE and autoradiography. Note that part of the envelope-bound pPORA is artificially processed as a result of UV-light treatment. (*C*) Identification of crosslink products by immunoprecipitation. Aliquots of the samples described in *B* were subjected to immunoprecipitation by using the indicated anti-Ptc130 (lane 6, corresponding to lane 3), anti-Ptc33 (lane 7, corresponding to lane 4), anti-Ptc16 (lane 7, corresponding to lane 5), and anti-Ptc52 (lane 9, corresponding to lane 5) antisera. Lanes 10–13 show that no precipitates are formed with respective preimmune sera (PIS).

the absence of added nucleoside triphosphates or in the presence of either 0.1 mM Mg-ATP (binding) or 0.1 mM Mg-ATP plus 0.1 mM Mg-GTP (insertion). After 15 min in darkness, the incubation mixtures were exposed to UV light to activate APDP, and the reaction terminated immediately thereafter (29). Then, protein extracts were prepared from mixed OM-IM fractions, reduced, separated by SDS/PAGE, and detected by autoradiography. As shown in Fig. 6*B*, several different crosslink products were obtained. Among them were Ptc130, Ptc52, Ptc33, and Ptc16, as demonstrated by immunoprecipitation with monospecific antisera (Fig. 6*C*). Their labeling pattern suggested a time course in which pPORA first interacted with Ptc130, then bound Ptc33 and Ptc16 and several other yet-unidentified envelope proteins, and only later approached Ptc52. The 47-kDa protein (Ptc47) was not photo-crosslinked but interacted with Ptc52, as deduced from the coimmunoprecipitation studies shown in Fig. 5*D*.

In summary, the results shown in this study demonstrate that multiple protein import complexes exist in barley chloroplasts. Moreover, the detection of Pchlide *b* as part of the PTC complex ultimately answers previous criticism on the presence of this pigment in etiolated plants (see ref. 30 for discussion), and therefore, our results further support the LHPP (light-harvesting POR-protochlorophyllide complex) model (45).

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