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Substrate-Dependent Transport of the NADPH:Protochlorophyllide Oxidoreductase into Isolated Plastids

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The key regulatory enzyme of chlorophyll biosynthesis in higher plants, the light-dependent NADPH:protochlorophyllide oxidoreductase (POR), is a nuclear-encoded plastid protein. Its post-translational transport into plastids is determined by its substrate. The precursor of POR (pPOR) is taken up and processed to mature size by plastids only in the presence of protochlorophyllide (Pchlide). In etioplasts, the endogenous level of Pchlide saturates the demands for pPOR translocation. During the light-induced transformation of etioplasts into chloroplasts, the Pchlide concentration declined drastically, and isolated chloroplasts rapidly lost the ability to import the precursor enzyme. The chloroplasts' import capacity for the pPOR, however, was restored when their intraplastidic level of Pchlide was raised by incubating the organelles in the dark with δ-aminolevulinic acid, a common precursor of tetrapyrroles. Additional evidence for the involvement of intraplastidic Pchlide in regulating the transport of pPOR into plastids was provided by experiments in which barley seedlings were grown under light/dark cycles. The intraplastidic Pchlide concentration in these plants underwent a diurnal fluctuation, with a minimum at the end of the day and a maximum at the end of the night period. Chloroplasts isolated at the end of the night translocated pPOR, whereas those isolated at the end of the day did not. Our results imply that the Pchlide-dependent transport of the pPOR into plastids might be part of a novel regulatory circuit by which greening plants fine tune both the enzyme and pigment levels, thereby avoiding the wasteful degradation of the imported pPOR as well as photodestruction of free Pchlide.

INTRODUCTION

The light-induced transformation of etioplasts to chloroplasts in higher plants is controlled by two different genetic systems. A minor part of plastid proteins is encoded by the plastid DNA (Sugiura, 1992), and their mRNAs are translated on plastid ribosomes (for reviews, see Mullet, 1988; van Grinsven and Kool, 1988; Reinbothe and Parthier, 1990; Reinbothe et al., 1993). The majority of plastid proteins, however, are encoded by nuclear genes (for reviews, see Harpster and Apel, 1985; Tobin and Silverthorne, 1985; Thompson and White, 1991; Susek and Chory, 1992) and are synthesized in the cytosol as larger precursor molecules (Keegstra et al., 1989; Archer and Keegstra, 1990). The continuous transport of these proteins from their cytosolic origin to their organellar destination plays an essential role in the maintenance of the plastid compartment. Thus far, this precursor protein import into plastids has been considered to be largely a housekeeping function of the plant cell (Boyle et al., 1986; de Boer et al., 1988). In this respect, it appears to differ from most other steps that are involved in regulating the synthesis and accumulation of nuclear-encoded chloroplast proteins, such as transcription, post-transcriptional RNA processing and modification, translation and post-translational protein assembly, and stabilization (for reviews, see Tobin and Silverthorne, 1985; Mullet, 1988; Thompson and White, 1991). However, more recent evidence suggests that plastid protein transport may also be under developmental control (Dahlin and Cline, 1991).

In this study, we investigated the post-translational transport of the precursor of nuclear-encoded NADPH:protochlorophyllide oxidoreductase (pPOR; EC 1.6.99.1) into plastids. This key enzyme of chlorophyll biosynthesis in higher plants exhibits several interesting features. First, maximal amounts of the protein are present in etioplasts of dark-grown seedlings, whereas light-grown seedlings contain only traces of POR (Apel, 1981; Batschauer and Apel, 1984; Griffiths et al., 1985; Mösinger et al., 1985; Forreiter et al., 1990; Benli et al., 1991). Second, POR's catalytic activity directly depends on light (Griffiths, 1975, 1978; Apel et al., 1980; Forreiter et al., 1990); however, it is as yet undetermined how light operates in enzyme catalysis. Third, during catalysis, POR is rapidly inactivated and subsequently degraded (Kay and Griffiths, 1983; Häuser et al., 1984; Forreiter et al., 1990).

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Based on these findings, one might ask whether light-grown seedlings continuously import the pPOR into their chloroplasts, which, after catalysis, would likely be destined for degradation, or whether some mechanism exists that controls the import of the precursor protein. In our study, we have addressed this question. Using a homologous barley system, we demonstrated that the transport of the pPOR into plastids is determined by its substrate, protochlorophyllide (Pchlide), emphasizing that protein transport may also be a specific regulatory step during light-induced chloroplast formation.

RESULTS

Differences in Transport of the pPOR into Etioplasts and Chloroplasts Reveal Pchlide as a Specific Requirement for the Import Pathway

Import studies were performed in a homologous in vitro system consisting of the pPOR, which was synthesized by coupling transcription of a *por*-specific barley cDNA (Schulz et al., 1989) and translation of the resulting mRNA in a wheat germ system, and isolated plastids (see Methods). In a time course experiment, we compared the ability of etioplasts and chloroplasts, which were prepared from dark-grown and light-grown barley seedlings, respectively, to import the ^{35}S-methionine–labeled 44-kD pPOR. To avoid a depletion of the intraplastidic energy sources assumed to be required for transport throughout the incubation, the assays were supplemented with ATP (see Methods). After various lengths of incubation, the assays were subjected to centrifugation (Reinbothe et al., 1990). The pPOR molecules that had not been sequestered by the plastids and thus remained in the supernatant fraction after centrifugation were precipitated with trichloroacetic acid and processed for PAGE as described previously (Reinbothe et al., 1990). Intact and broken plastids present in the sediment fraction after centrifugation were separated by flotation on Percoll cushions (Grossman et al., 1982). After resedimentation, the intact plastids were treated with thermolysin (Cline et al., 1984), followed by their repurification as described previously. Proteins were prepared from the plastids by sonication and precipitation with trichloroacetic acid. After electrophoresis in separate polyacrylamide gradients (Laemmli, 1970), the ^{35}S-methionine–labeled pPOR and POR, which were found in the supernatant and the thermolysin-treated plastids, respectively, were detected by autoradiography.

Figure 1 shows that etioplasts and chloroplasts differed markedly in their capability to sequester the pPOR. In the presence of etioplasts, the pPOR rapidly disappeared from the supernatant fraction. Simultaneously, the mature 36-kD POR appeared in the protease-treated etioplasts. Within the first 5 min of the incubation, uptake and processing of the precursor protein were completed (Figure 1A). In contrast, no precursor translocation could be detected in assays containing isolated intact chloroplasts: the level of the precursor polypeptide remained constant, and no mature enzyme appeared inside the plastids (Figure 1A). Because in this first experiment the incubations with etioplasts were performed in the dark, whereas those with chloroplasts were performed in the light, one could argue that light might have inhibited the import of the pPOR into chloroplasts. However, the transport of the pPOR into etioplasts occurred both in the dark and in the light (Figure 1B). Conversely, import of the pPOR into chloroplasts was not observed under either condition (Figure 1B).

Figure 2 demonstrates that this effect seemed to be specific for the pPOR because the precursors for other nuclear-encoded plastid proteins, such as the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase of barley and a chimeric precursor protein consisting of the transit peptide of plastocyanin of *Silene pratensis* fused to cytosolic dihydrofolate reductase of mouse (Hageman et al., 1990), were imported into both chloroplasts and etioplasts.

One possible explanation for the obvious differences of etioplasts and chloroplasts in importing the pPOR might be that both plastid types contain different amounts of a cofactor

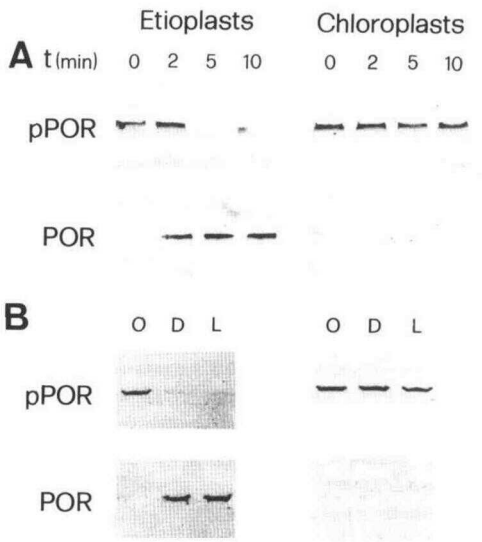

Figure 1. Plastid Import and Processing of the pPOR.

(A) Time course analysis of pPOR and POR protein levels during the incubations with etioplasts and chloroplasts prepared from dark-grown and light-grown barley seedlings, respectively. After the various periods of time (t) indicated, the ^{35}S-methionine–labeled pPOR molecules that had not been sequestered by the plastids and thus remained in the supernatant after centrifugation of the assays were precipitated with trichloroacetic acid, separated electrophoretically, and detected by autoradiography. Similarly, mature radiolabeled POR was recovered from thermolysin-treated intact plastids by sonication and precipitation with trichloroacetic acid, run in a separate denaturing polyacrylamide gradient, and detected by autoradiography. Only the relevant parts of the autoradiograms, exposed for an identical period, are shown.

(B) Time course analysis of pPOR and POR abundances before (0) and after a 15-min-dark (D) or -light (L) incubation with etioplasts and chloroplasts, which were prepared and treated as given in **(A)**.

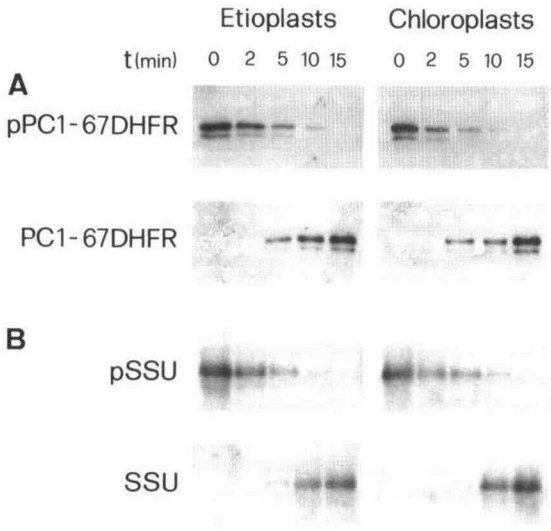

Figure 2. Post-Translational Import of Authentic and Artificial Precursor Proteins into Isolated Barley Plastids.

(A) Time course of the levels of the precursor (pPC1-67DHFR) and mature (PC1-67DHFR) forms of an in vitro–synthesized chimeric protein containing the chloroplast transit peptide of plastocyanin from *S. pratensis* fused at the N terminus to the cytosolic dihydrofolate reductase of mouse.

(B) Time course of the levels of the precursor (pSSU) and mature (SSU) forms of ribulose-1,5-bisphosphate carboxylase/oxygenase. Details of the incubation of the ^{35}S-methionine–labeled proteins with the plastids are as given in Figure 1.

specifically required for the translocation of this particular precursor protein. One such factor might be Pchlide, which is the enzyme's substrate. This pigment is found in significant amounts only in etioplasts, whereas in the course of light-induced chloroplast differentiation, its concentration declines below the limit of detection (Beale and Weinstein, 1990). To evaluate this possibility, we investigated the effect of Pchlide added exogenously to assays containing chloroplasts. As shown in Figure 3, the addition of exogenous Pchlide did, in fact, restore the transport of the pPOR into chloroplasts. At Pchlide concentrations exceeding 10 μM, the pPOR was taken up and processed to mature size by chloroplasts as efficiently as by etioplasts incubated in the absence of the pigment (see Figure 1). Figure 4 shows that the imported and processed POR was similarly targeted to the membrane fractions inside the etioplasts and Pchlide-incubated chloroplasts.

Attempts to Determine the Site at Which Pchlide Operates in pPOR Transport into Plastids

At first glance, the results presented thus far seem to indicate that Pchlide induces the translocation of the pPOR outside the isolated chloroplasts either by binding to the precursor protein and transferring it into a translocation-competent form or by interacting with components of the import machinery that are exposed on the outer surface of the chloroplast envelope. Both explanations, however, are not very likely if one considers the experiments with etioplasts, which demonstrate the import of the pPOR in the absence of exogenously added Pchlide (see Figure 1). One way to reconcile the results obtained for chloroplasts with those reported for etioplasts might be to postulate that Pchlide is exported specifically from the etioplasts in a manner similar to that demonstrated for other tetrapyrrole derivatives, such as protoporphyrinogen IX (Lehnen et al., 1990), phytochromobilin (Terry and Lagarias, 1991), and heme (Thomas and Weinstein, 1990). Such an export mechanism, however, has not been demonstrated for Pchlide.

Alternatively, Pchlide might be released from etioplasts broken throughout the incubation and could substitute for the Pchlide supplied exogenously in facilitating the translocation of the pPOR. However, when Pchlide was measured in the assay medium after incubation of isolated etioplasts, its concentration was at least 500-fold lower than that required exogenously for the uptake of the pPOR into isolated chloroplasts (data not shown). Thus, it is not very likely that Pchlide operated in pPOR translocation from the outside of the plastids either by binding to the precursor or by interacting with components of the import machinery that are exposed on the outer surface of the plastid envelope. Instead, a small fraction of the exogenous Pchlide might have diffused into the isolated chloroplasts and subsequently might have triggered the uptake of the pPOR by operating from the inside of the plastids. Figure 5B shows that chloroplasts that had been preincubated with Pchlide and had then been washed to remove the excess of

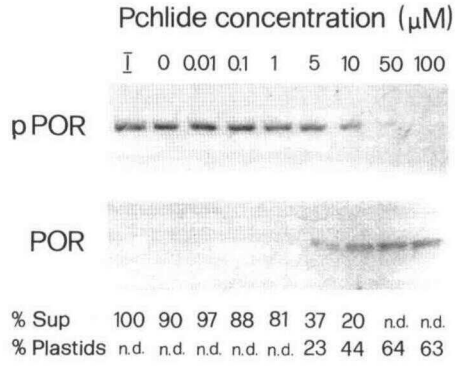

Figure 3. Effect of Pchlide on the Transport of the pPOR into Isolated Chloroplasts of Barley.

Isolation and incubation of isolated chloroplasts were performed in the dark as described in Figure 1. Pchlide, which was added to the assays at the indicated concentrations, was isolated and purified as described in Methods. The numbers below the autoradiograms indicate the percentages of the pPOR in the supernatant fraction (Sup) or of mature POR in the protease-treated plastids relative to the initial level of the pPOR (I), as determined by scanning the optical density of the signals on the autoradiograms. n.d. designates pPOR or POR levels that are not detectable.

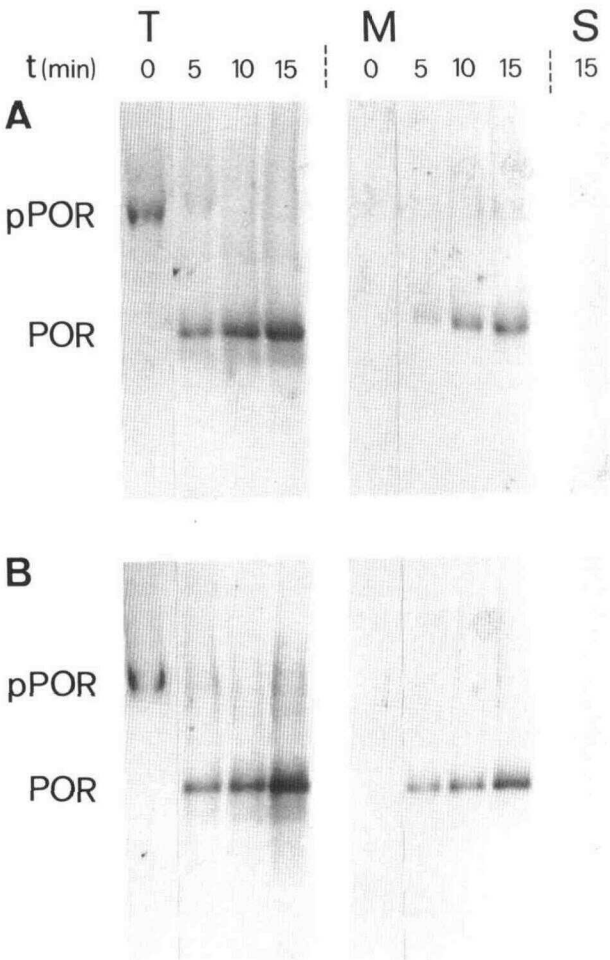

Figure 4. Post-Import Routing of POR inside Etioplasts and Chloroplasts.

(A) Etioplasts were prepared from dark-grown barley seedlings and incubated with the in vitro–synthesized, radiolabeled pPOR for the indicated time (t) periods. After the import reaction, one-half of the etioplasts were treated with thermolysin and further fractionated into stroma (S), membranes (M), and envelopes (not shown). The other half of the etioplasts were left untreated (T). The autoradiogram shows the levels of the pPOR and POR in the etioplasts (T) and their different suborganellar fractions (M and S).

(B) Chloroplasts were prepared from light-grown seedlings and incubated with the radiolabeled pPOR in assays supplemented with exogenously added Pchlide, as described in Figure 3. Chloroplasts were then treated without (T) or with (M and S) thermolysin and further fractionated into membranes (M) and stroma (S), as given in **(A)**. The autoradiogram shows the levels of the pPOR and POR in the chloroplasts (T) and their different suborganellar fractions (M and S).

the exogenously applied pigment indeed retained the capacity to import the pPOR in the absence of additional Pchlide. Chloroplasts that had not been pretreated with Pchlide could sequester the pPOR only in the presence of the added pigment (Figure 5C). Together, these experiments suggested but did not prove that endogenous Pchlide was required for pPOR translocation.

To show ultimately that intraplastidic but not enzyme-bound Pchlide is required for pPOR transport into plastids, the following approaches were chosen. First, the endogenous level of Pchlide was manipulated in chloroplasts by incubating the organelles in vitro with the pigment precursor δ-aminolevulinic acid (δ-ALA) in the dark (Gomez-Silva et al., 1985a). As assessed by its fluorescence emission shown in Figure 6, Pchlide accumulated in a linear fashion during a 15-min incubation as compared with control chloroplasts not fed with δ-ALA (data not shown). Second, pPOR–pigment complexes were enriched by centrifugation through Sephadex G-25 columns (Olsen et al., 1989). The pPOR was incubated with either Pchlide or Pchlide and NADPH so that the formation of enzyme–substrate complexes could occur. As shown in Figure 7A, the pPOR–Pchlide complex could be detected spectrometrically in the flowthrough by its blue light–induced fluorescence emission peak at 628 nm. When Pchlide was added to wheat germ extracts that had not been programmed with *por* mRNA and thus lacked the pPOR, no Pchlide could be traced in the flowthrough after centrifugation (Figure 7A), emphasizing that only the precursor protein–bound pigment was recovered by this separation. When NADPH and Pchlide were added to pPOR-containing assays during a dark preincubation, a ternary pPOR–Pchlide–NADPH complex was formed (Figure 7B). After centrifugation,

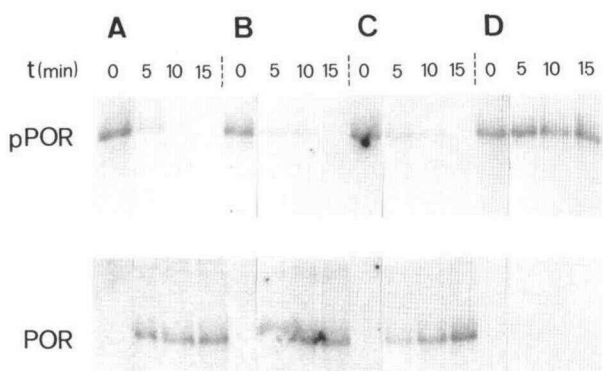

Figure 5. Effect of Pretreating Chloroplasts with Pchlide on the Subsequent Import of the pPOR.

Chloroplasts were preincubated with or without Pchlide and subsequently washed to remove the excess pigment. The different chloroplasts were then incubated with the in vitro–synthesized pPOR in assays containing or lacking additional Pchlide (15 μM final concentration). The autoradiograms show time (t) courses of the levels of the pPOR and POR in the following assays.

(A) Chloroplasts pretreated with Pchlide and incubated with the pPOR in the presence of additional pigment.

(B) Pchlide-pretreated chloroplasts incubated with the pPOR in the absence of additional pigment.

(C) Chloroplasts not pretreated with Pchlide but incubated with the pPOR in the presence of added pigment.

(D) Chloroplasts neither pretreated nor incubated with Pchlide.

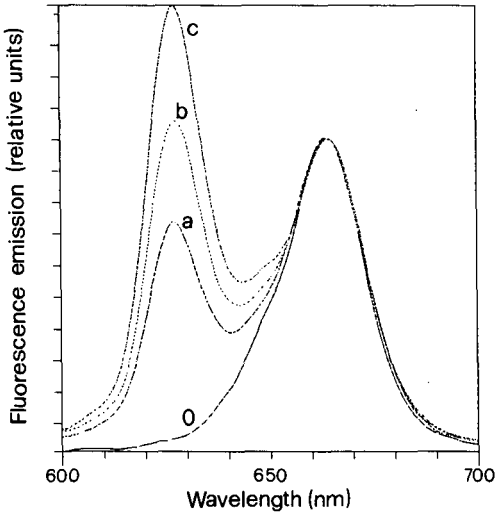

Figure 6. Pchlide Formation in Isolated Chloroplasts of Barley.

Chloroplasts were incubated with δ-ALA for 0 (0), 5 (a), 10 (b), or 15 (c) min in the dark. The curves, normalized at a wavelength of 665 nm, indicate arbitrary units. Pchlide and chlorophyll were detected by their fluorescence emission maxima at 628 and 665 nm, respectively, at an excitation wavelength of 431 nm, using a spectrometer.

this ternary complex was able to convert Pchlide to chlorophyllide (Chlide) when illuminated (Figure 7B). Ternary complexes exposed to light before centrifugation were recovered as Chlide-containing complexes (Figure 7C), suggesting that at least the pigment product of catalysis remains bound to the enzymatically active precursor protein.

For the in vitro transport studies, chloroplasts containing or lacking the exogenous δ-ALA-derived Pchlide were incubated with the pPOR–Pchlide, pPOR–Pchlide–NADPH, or pPOR–Chlide complexes described previously. As a control, the "naked" pPOR, that is, the polypeptide lacking Pchlide, Chlide, and NADPH, was used. Figure 8 demonstrates that the pPOR was taken up by chloroplasts only when they contained Pchlide. Chloroplasts lacking this pigment were not able to import the pPOR. A similar conclusion could be drawn for assays containing either the binary pPOR–Pchlide or ternary pPOR–Pchlide–NADPH complexes (Figure 8). In both cases, pPOR translocation occurred only into chloroplasts containing Pchlide. In contrast, the pPOR–Chlide complex could not be sequestered by chloroplasts either containing or lacking Pchlide (Figure 8). Apparently, the pPOR–Chlide complex was not competent for transport across the chloroplast envelope membranes.

There are at least two possible ways to explain how Pchlide could regulate the transport of the pPOR into etioplasts and δ-ALA–fed chloroplasts. Taking into account the previous findings of Joyard et al. (1990, 1991) that Pchlide synthesis is likely to occur within the plastid envelope, we concluded that this pigment might either cause the pPOR to bind to the plastid

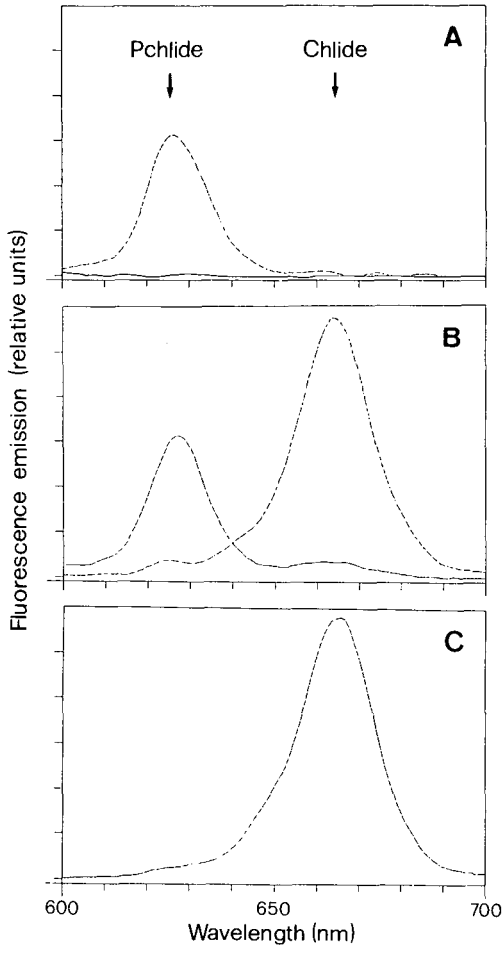

Figure 7. Purification of pPOR–Pigment Complexes by Centrifugation through Sephadex G-25 Columns.

pPOR–pigment complexes were formed by incubating the in vitro–synthesized pPOR either with Pchlide in the dark or with both NADPH and Pchlide in the dark or in the light. The different pPOR complexes were loaded onto Sephadex G-25 columns equilibrated in the import buffer. As a control, Pchlide added to wheat germ extracts that had not been programmed with *por* mRNA was loaded onto a separate column. After centrifugation, part of the assay containing the pPOR–Pchlide–NADPH complex was kept in the dark, whereas another part was exposed to light, causing Chlide formation. Pchlide and Chlide were detected by their fluorescence emission maxima at 628 and 665 nm, respectively, at an excitation wavelength of 431 nm. The different curves show fluorescence emission spectra of the following pPOR–pigment complexes.

(A) pPOR–Pchlide complex (upper curve) as compared with free Pchlide removed during gel filtration (lower curve).

(B) pPOR–Pchlide–NADPH complex recovered after gel filtration (left peak) as compared with the pPOR–Chlide complex produced by exposing the gel-filtered pPOR–Pchlide–NADPH complex to light (right peak).

(C) pPOR–Chlide complex formed by illuminating the pPOR–Pchlide–NADPH complex prior to gel filtration and removing the excess Pchlide by gel filtration.

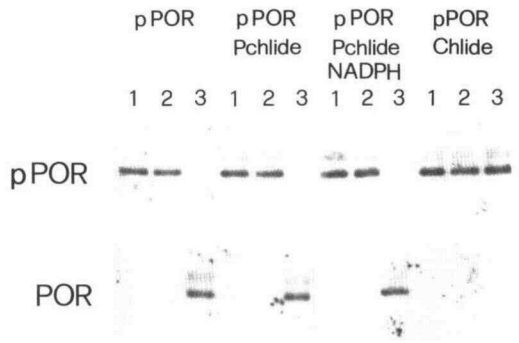

Figure 8. Effect of Substrates and Products on Transport of the pPOR into Chloroplasts.

After a 15-min preincubation with δ-ALA in the dark, chloroplasts were sedimented by centrifugation and resuspended in the import buffer containing either the radiolabeled pPOR or the indicated pPOR–pigment complexes. The autoradiograms show the levels of the pPOR and POR before (lane 1) or after a 15-min incubation in the dark with chloroplasts lacking (lane 2) or containing (lane 3) Pchlide.

envelope or trigger its subsequent translocation across the envelope membranes. To distinguish between these two possibilities, the following experiment was performed. Chloroplasts were first incubated with δ-ALA in the dark to induce intraplastidic Pchlide formation. Controls were fed with phosphate buffer alone instead of δ-ALA. Subsequently, the chloroplasts were depleted of ATP by keeping them on ice for 1 hr. The gel-filtered pPOR was then added to Pchlide-containing or Pchlide-free chloroplasts in assays lacking ATP. All subsequent incubations were performed at 4°C to allow the recovery of pPOR molecules that might have bound to, but were not imported into, the chloroplasts (Olsen et al., 1989). As shown in Figure 9, intraplastidic Pchlide was insufficient to cause binding of the pPOR to the plastid envelope. This can be seen from the constant level of the pPOR in the supernatant and the lack of mature POR in the plastid fraction (Figure 9, lane 3). ATP, however, was able to cause the binding of the precursor protein to the outer envelope of Pchlide-free chloroplasts. This is evident from the quantitative shift of the pPOR from the supernatant to the plastid fraction (Figure 9, lane 4). In the absence of ATP, the pPOR did not bind to either Pchlide-free or Pchlide-containing chloroplasts (Figure 9, lanes 2 and 3). If Pchlide-containing chloroplasts were incubated with the pPOR in assays supplemented with ATP, the precursor protein was imported into the chloroplasts (Figure 9, lane 5). This experiment thus shows that intraplastidic Pchlide is required for the actual translocation step by which the pPOR crosses the plastid envelope membranes, but the pigment is dispensable for the initial binding of the precursor protein to the plastids. The latter step, which requires ATP, appears to be mediated by a protease-sensitive component at the outer surface of the chloroplasts. Thermolysin pretreatment of chloroplasts containing or lacking Pchlide led to a complete loss of binding of the pPOR to the outer plastid envelope and thus abolished the entire transport process (Figure 9, lanes 6 and 7).

In Vivo Evidence for the Pchlide-Dependent Import Pathway

Based on the result that intraplastidic Pchlide is required for the translocation of the pPOR across the plastid envelope membranes, we attempted to find conditions under which the endogenous level of the pigment in vivo correlates with the plastids' import competence for the pPOR. Seedlings were grown under a 10-hr-light/14-hr-dark cultivation regime for 5 days. Chloroplasts were isolated from plants that had been harvested either at the end of the light or at the end of the dark period, and the levels of Pchlide were determined spectrometrically. Chloroplasts isolated from plants at the end of the dark period accumulated a small but significant level of Pchlide (data not shown; see also, for example, Griffiths et al., 1985). In contrast, chloroplasts isolated from plants that had been harvested at the end of the light period did not accumulate Pchlide to detectable levels. In parallel to the spectroscopic analyses, the transport competence of the different plastids was investigated with respect to the pPOR. As shown in Figure 10B, chloroplasts that had been harvested at the end of

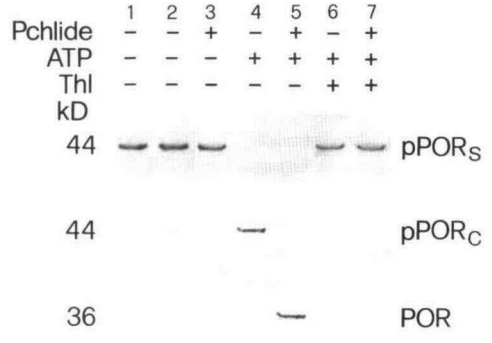

Figure 9. Chloroplast Receptor Binding Characteristics of the pPOR.

Chloroplasts lacking (–) or containing (+) Pchlide produced by δ-ALA feeding were depleted of ATP and subsequently incubated with the gel-filtered radiolabeled pPOR. As indicated, the assays were supplemented with ATP (5 mM final concentration). In some cases, chloroplasts were treated with thermolysin (Thl) prior to their addition to the gel-filtered pPOR (lanes 6 and 7). After a 15-min-dark incubation on ice, proteins were recovered from the supernatant and the chloroplast fractions, which were obtained after centrifugation, as described in Figure 1. The only modification was that only those plastids that had been used for the entire transport reaction, including the binding of the pPOR to and its subsequent translocation across the plastid envelope membrane, were treated with thermolysin (lane 5). The autoradiograms show the 44-kD pPOR present in either the supernatant ($pPOR_S$) or the chloroplast ($pPOR_C$) fractions as well as the mature, 36-kD POR before (lane 1) and after (lanes 2 to 7) the incubation.

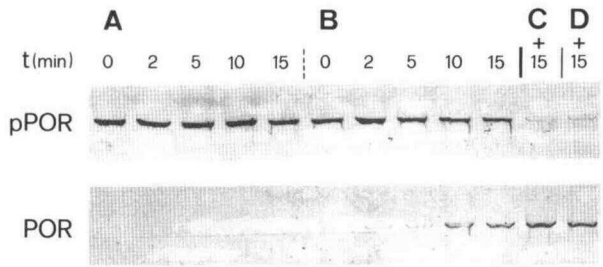

Figure 10. Import of the pPOR into Chloroplasts of Barley Seedlings Grown in a 10-hr-Light/14-hr-Dark Regime.

Chloroplasts were isolated from plants harvested either at the end of the light or at the end of the dark period and incubated with the radiolabeled pPOR in assays supplemented without or with (+) isolated Pchlide. After various time (t) intervals, the levels of the pPOR and POR were determined in the supernatant and plastid fractions, as described in Figure 1.

(A) and **(B)** Time courses of the levels of the pPOR and POR in assays containing chloroplasts from plants harvested either at the end of the light or at the end of the dark periods, respectively.

(C) and **(D)** The time courses are as given for **(A)** and **(B)**, but they show the levels of the pPOR and POR in assays supplemented with Pchlide.

the dark period were able to import the pPOR. In contrast, chloroplasts isolated at the end of the light period did not sequester the pPOR (Figure 10A). However, their transport capacity for the pPOR could be restored by the exogenous application of isolated Pchlide (Figure 10C). Similarly, exogenous Pchlide also stimulated the import of the pPOR into chloroplasts harvested at the end of the dark period (Figure 10D), suggesting that the intraplastidic level of Pchlide had not fully saturated the demands for the translocation of the precursor protein.

DISCUSSION

Intraplastidic Pchlide Is Required for the Translocation of the pPOR across the Plastid Envelope Membranes

An important result of our work is the demonstration of a novel transport mechanism by which a nuclear-encoded plastid protein is imported into the organelle. We have shown that the pPOR of barley, synthesized in vitro from a corresponding full-length cDNA, is taken up and processed to mature size by isolated plastids only when they contain Pchlide. During their light-induced transformation into chloroplasts, etioplasts convert Pchlide to Chlide and simultaneously lose the capability to sequester the pPOR (S. Reinbothe, unpublished data). Chloroplasts thus are no longer able to import the pPOR, although they efficiently sequester other plastid precursor proteins, such as the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase and a chimeric protein consisting of the chloroplast transit peptide of plastocyanin of *S. pratensis* linked to the cytosolic dihydrofolate reductase of mouse. Thus, differing intraplastidic ATP levels, which might have arisen from different rates of uptake by the ATP/ADP translocator (Robinson and Wiskich, 1977; Flügge and Heldt, 1991) of ATP added exogenously to all of the assays used in this study, did not account for the obvious difference between etioplasts and chloroplasts in importing the pPOR. The chloroplasts' import capacity for the pPOR could be restored either by the endogenous production of Pchlide, caused by feeding the chloroplasts with δ-ALA or by cultivating seedlings in light/dark cycles, or by the exogenous application of Pchlide. Pchlide dependency on protein import appears to be specific for the pPOR. It does not seem to be part of a general developmental regulation of the import apparatus that might depend on the age or the type of plastids, as discussed previously by Chitnis et al. (1986, 1987, 1988), Halpin et al. (1989), and Dahlin and Cline (1991) for the import of other nuclear-encoded plastid proteins.

Recently, the import of a wheat pPOR into isolated pea chloroplasts has been shown to occur in the absence of added Pchlide (Teakle and Griffiths, 1993). The seeming discrepancy between this result and our work can be explained by the finding shown in Figure 10. Seedlings of barley and other plant species that are grown under dark/light cycles reaccumulate Pchlide within their chloroplasts during the night (dark) period (see, for example, Griffiths et al., 1985). This pigment, in turn, may trigger the translocation of the pPOR into isolated plastids, as shown in our study. Teakle and Griffiths (1993) cultivated pea seedlings in a 12-hr-light/12-hr-dark regime, and thus it seems likely that trace amounts of endogenous Pchlide, which were still present in the plastids after their isolation, might have triggered the uptake of the pPOR.

Considerations on the Mechanism of the Pchlide-Dependent Transport of the pPOR into Plastids

The import of the pPOR into plastids requires intraplastidic Pchlide and ATP and is mediated by a yet undetermined receptor protein component exposed to the outer surface of the plastids. Because Pchlide synthesis has been shown to occur within the plastid envelope (Joyard et al., 1990, 1991), it was not unexpected to find that Pchlide triggered the actual transfer of the pPOR across the plastid envelope membranes. No evidence was obtained that intraplastidic Pchlide is needed for the binding of the pPOR to the plastid envelope. Pchlide binding to the pPOR outside the plastids prior to transport did not induce the import of the precursor protein into chloroplasts lacking this pigment. However, substrate binding also did not interfere with the transport of the pPOR into δ-ALA–fed and thus Pchlide-containing chloroplasts: binary pPOR–Pchlide or ternary pPOR–Pchlide–NADPH complexes were translocated across the plastid envelope membranes with efficiencies similar to that of the pPOR lacking Pchlide. In contrast, enzymatic Chlide formation completely blocked subsequent transloca-

tion of the pPOR into δ-ALA–fed chloroplasts. Chlide remained bound to the pPOR and appeared to stabilize a transport-incompetent conformation of the precursor protein. This finding suggests that the pPOR has to adopt an unfolded conformation so that its subsequent translocation across the outer and inner plastid envelope membranes can occur.

By analogy with numerous other proteins that have to cross biological membranes, such as the endoplasmic reticulum (for reviews, see Verner and Schatz, 1988; Rapoport, 1992), the inner and outer mitochondrial membranes (for reviews, see Eilers and Schatz, 1986, 1988; Eilers et al., 1988; Pfanner and Neupert, 1990; Glick and Schatz, 1991), and the plastid envelope membranes (della-Cioppa and Kishore, 1988; Pilon et al., 1992a, 1992b), we propose that the pPOR becomes unfolded either before or during translocation. Ligands, such as substrates or their products, thus would have to dissociate from the pPOR to allow the precursor protein to attain a transport-competent, unfolded conformation. Chlide, but not Pchlide and NADPH, apparently impaired this unfolding of the pPOR. By analogy, high-affinity ligand binding, such as binding of the substrate analog *N*-(phosphonomethyl)glycine to 5-*enol*pyruvylshikimate-3-phosphate synthase (della-Cioppa and Kishore, 1988) or of methotrexate to dihydrofolate reductase used as a passenger protein (Eilers and Schatz, 1986; Eilers et al., 1988), has been demonstrated to induce tight folding of the precursor proteins and thus to inhibit their import into chloroplasts and mitochondria, respectively. In contrast, the binding of substrates to the organellar proteins, such as phospho*enol*pyruvate and/or shikimate-3-phosphate to 5-*enol*pyruvylshikimate-3-phosphate synthase (della-Cioppa and Kishore, 1988), or the attachment of prosthetic groups to enzymes, such as flavin adenine dinucleotide to ferredoxin:$NADP^+$ oxidoreductase (Carillo, 1985), did not influence subsequent precursor translocation into chloroplasts.

What Is the Driving Force for the Translocation of the pPOR into the Plastids?

There are at least two modes by which Pchlide might regulate the translocation of the pPOR across the plastid envelope membrane. First, Pchlide might interact with an internal component of the transport machinery exposed to the inner side of the plastid envelope. Alternatively, it might bind to the precursor enzyme and thus could cause refolding plus movement of the pPOR into the plastids.

If the latter mode of pPOR translocation into plastids proves to be correct, it could be compared with the "nonconservative" import pathways (for nomenclature, see Pfanner and Neupert, 1990; Pfanner et al., 1992) by which the various cytochromes enter mitochondria (Nicholson and Neupert, 1989; Glick et al., 1992; Wachter et al., 1992). For example, the transport of cytochrome *c* into the intermembrane space of mitochondria has been shown to require the covalent attachment of heme in its reduced form to the apoprotein (Nicholson and Neupert, 1989). Similarly, the apoproteins of cytochromes c_1 and b_2 bind reduced heme after their entry into the intermembrane space but prior to their final insertion into the mitochondrial inner membrane (Ohashi et al., 1982; Wachter et al., 1992). In all of these cases, the covalent attachment of heme is catalyzed by enzymes with cytochrome *c* heme lyase–like activities (Lill et al., 1992). In this respect, the various cytochromes differ from plastid POR, which is thought to bind its substrates spontaneously and noncovalently (Griffiths, 1978; Apel et al., 1980; S. Reinbothe, unpublished data). Furthermore, the apoproteins of cytochromes c_1 and b_2, but not of cytochrome *c*, contain cleavable N-terminal bipartite signal sequences that are involved in the proper targeting and insertion of the precursor proteins into the inner mitochondrial membrane (van Loon et al., 1986, 1987; Glick et al., 1992; Wachter et al., 1992). In contrast, the pPOR is directed via its N-terminal transit peptide to the inside of Pchlide-containing plastids, where its processing to the mature size and subsequent routing to its final destination occur. Taken together, these results demonstrate that, although similar in several respects, the import routes of the various cytochrome apoprotein precursors and the pPOR into mitochondria and plastids, respectively, are distinct in most regards. This highlights the importance of the novel plastid import mechanism described in this work.

Functional Implications of the Pchlide-Dependent Import Pathway

The results of our work have two interesting implications. First, they suggest that the Pchlide-controlled import of the pPOR might prevent a wasteful proteolytic degradation of the mature enzyme within the plastid compartment. Supporting this idea, POR loaded with Pchlide has previously been shown to be resistant to proteolytic attack, whereas POR devoid of its substrate was found to be rapidly degraded (Kay and Griffiths, 1983; Häuser et al., 1984). Second, our results imply a novel regulatory circuit by which greening plants protect their plastids from photooxidation, a phenomenon dark-grown plants have to cope with upon illumination (Tripathy and Chakraborty, 1991; Chakraborty and Tripathy, 1992). Pchlide has recently been shown to be synthesized in the plastid envelope (Joyard et al., 1990, 1991). This site of synthesis may ensure that freshly formed Pchlide molecules immediately bind to the pPOR and are subsequently targeted to the stroma and the thylakoid membranes. At either of these locations, the reduction of Pchlide could ultimately occur, followed by binding of the resulting chlorophyll(ide) to the different chlorophyll *a*/*b* binding proteins. By lowering its actual concentration, free Pchlide thus might be protected against interaction with molecular oxygen and could not cause the formation of highly reactive oxygen radicals and singlet oxygen that ultimately damage the plastid compartment (Tripathy and Chakraborty, 1991; Chakraborty and Tripathy, 1992).

The operation of this protection mechanism during light-induced greening would depend on a continuous surplus of nonprocessed POR precursor molecules that remain attached

to the outside of the plastids and are ready to interact with the freshly formed Pchlide. Indeed, recent work in our laboratory has shown that the pPOR accumulates in a protease-sensitive form at the outer surface of the plastids when dark-grown seedlings are exposed to light for 4 to 8 hr. Under these conditions, the very low intraplastidic Pchlide level limited precursor translocation. However, feeding the plastids δ-ALA increased the intraplastidic Pchlide level and simultaneously chased the arrested pPOR molecules into the organelles, where they appeared in the mature, protease-resistant form (S. Reinbothe and K. Apel, manuscript in preparation).

METHODS

Plant Growth

Seeds of barley (*Hordeum vulgare* cv Carina) were germinated on moist vermiculite either in complete darkness or in continuous white light (30 W/m^2, provided by fluorescent bulbs) at 23°C for 5 days or were cultivated in alternating 10-hr-light/14-hr-dark cycles.

Plastid Isolation and Post-Translational Import of in Vitro–Synthesized Precursor Proteins

Primary leaves were cut from the seedlings, and 5-cm segments beginning 1 cm from the leaf tip were used for plastid isolation. Etioplasts and chloroplasts from dark-grown and light-grown seedlings, respectively, were enriched by Percoll (Pharmacia-LKB, Sweden) gradient centrifugation (Gomez-Silva et al., 1985b) and further purified by flotation on Percoll cushions (Grossman et al., 1982), as described previously (Reinbothe et al., 1990, 1993). Plastid preparations yielded almost homogeneous populations of intact plastids, as assessed by phase-contrast microscopy.

For the import studies with the in vitro–synthesized precursor proteins (see the following discussion), 5 × 10^7 plastids per 50-μL assay, determined with a hemocytometer, and the import buffer of della-Cioppa et al. (1986) were used. If not stated otherwise (see text), all assays contained ATP at a final concentration of 5 mM. For the experiment described in Figure 8, the chloroplasts were first incubated with δ-aminolevulinic acid (δ-ALA; 0.5 mM final concentration) at 25°C in the dark for 15 min to induce intraplastidic protochlorophyllide (Pchlide) formation (Gomez-Silva et al., 1985a). Similarly, chloroplasts to be used in the experiment described in Figure 9 were first fed δ-ALA but were also kept on ice for 1 hr to deplete them of ATP (Theg et al., 1989). In contrast, chloroplasts to be used in the experiment described in Figure 5 were first incubated with isolated Pchlide (15 μM) in the dark for 15 min to allow diffusion of the exogenously applied pigment into the plastids to occur. Pchlide-free or Pchlide-containing plastids were then added to the different precursor of NADPH:protochlorophyllide oxidoreductase (pPOR)–pigment complexes purified by gel filtration (see the following discussion), as indicated in the text.

After various incubation times of the plastids at 25°C without or with the exogenously added isolated Pchlide (see text), the assays were diluted with Hepes buffer III (Reinbothe et al., 1990). After centrifugation at 10,000 rpm in a Sorvall RC-5B centrifuge (Du Pont de Nemours, Hamburg, Germany) at 4°C, using an HB4 rotor, proteins contained in the supernatant were precipitated with trichloroacetic acid (5% [w/v] final concentration) and were processed for PAGE as described previously (Reinbothe et al., 1990). The plastid sediment obtained after centrifugation was repurified by flotation on Percoll cushions (see the previous discussion), and the recovered intact plastids were treated with thermolysin according to Cline et al. (1984). After their repurification, the plastids were ruptured by sonication. Plastid proteins were precipitated with trichloroacetic acid from the plastid homogenate, as described previously (Reinbothe et al., 1990).

For the experiment described in Figure 4, the in vitro–synthesized radiolabeled pPOR was first incubated with either etioplasts or chloroplasts in assays supplemented without or with isolated Pchlide, respectively, as described previously. After various incubation times, one aliquot of the assays was centrifuged, and the plastids were treated with thermolysin (see the previous discussion). Another aliquot of the assays was left untreated, and proteins found in such total assays were precipitated with trichloroacetic acid. Plastids that had been treated with thermolysin were further fractionated into stroma, envelopes, and membranes, as described by Li et al. (1991). Proteins present in the stroma and membrane fractions of either etioplasts or chloroplasts were recovered by precipitation with trichloroacetic acid, as described previously.

Preparation of RNA, in Vitro Translation, and Immunodetection of Proteins

The mRNAs for the precursors of the POR of barley and of a chimeric protein, pPC1-67DHFR containing the chloroplast transit peptide of plastocyanin of *Silene pratensis* linked by a stretch of 10 amino acids (LQVDSRGSSG) to the complete cytosolic dihydrofolate reductase of mouse, were synthesized by in vitro transcription (Krieg and Melton, 1984) of corresponding plasmid clones, as specified by Schulz et al. (1989) and Hageman et al. (1990), respectively. High molecular mass RNA containing the mRNA for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase was prepared from light-grown barley seedlings as described previously (Reinbothe et al., 1990), and the poly(A)$^+$ RNA fraction was further enriched by messenger paper (Hybond-mAP; Amersham Int.) affinity chromatography (Werner et al., 1984).

For in vitro translation of proteins with L-^{35}S-methionine (0.37 MBq per 25-μL assay, 37 TBq/mmol; Amersham Int.) in a wheat germ system (Erickson and Blobel, 1983), suboptimal concentrations of *por*, pPC1-67DHFR, and poly(A)$^+$ RNAs were used (Reinbothe et al., 1990). All in vitro translations were performed in wheat germ extracts lacking endogenous POR activity. Thus, any detectable POR activity could be due only to the activity of the cDNA-encoded pPOR. The small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase was identified among total in vitro translation products of poly(A)$^+$ RNA by immunoadsorption to protein A–Sepharose (Pharmacia-LKB) with monospecific antibodies raised against the purified barley protein (Reinbothe et al., 1990).

Enrichment of pPOR–Pigment Complexes

After terminating the translation of *por* mRNA synthesized in vitro by the addition of an excess of L-methionine (8.3 mM final concentration), the formed pPOR was incubated with either Pchlide (15 μM) or Pchlide plus NADPH (0.5 mM) in the dark or in the light for 15 min. Control assays contained doubly distilled water instead of Pchlide or Pchlide plus NADPH. After the incubation, the assays were loaded onto disposable 1-mL columns packaged by repeated centrifugations at 3000

rpm for 4 min each in a Sorvall RC-5B centrifuge, using an HB4 rotor, with Sephadex G-25 (Pharmacia-LKB) equilibrated in the import buffer (see previous discussion). After dark centrifugation, the pPOR–Pchlide, pPOR–Pchlide–NADPH, or pPOR–Chlide complexes were detected in the flowthrough by their blue light–induced fluorescence emission at 628 and 665 nm, respectively, using a fluorescence spectrometer (model LS50; Perkin-Elmer).

Electrophoretic Techniques

Denaturing 11 to 20% (w/v) polyacrylamide gradients containing SDS were prepared according to Laemmli (1970). Except for the experiment described in Figure 4, the ^{35}S-methionine–labeled proteins found in the supernatant and sediment fractions after centrifugation of the import assays at the end of the incubations (see previous discussion) were run in separate gels and detected by autoradiography.

Miscellaneous

Pchlide was prepared from dark-grown barley seedlings, as described by Griffiths (1978).

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