Enzymatic Product Formation Impairs Both the Chloroplast Receptor-binding Function as Well as Translocation Competence of the NADPH: Protochlorophyllide Oxidoreductase, a Nuclear-encoded Plastid Precursor Protein

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Abstract. The key enzyme of chlorophyll biosynthesis in higher plants, the light-dependent NADPH:protochlorophyllide oxidoreductase (POR, EC 1.6.99.1), is a nuclear-encoded plastid protein. Its posttranslational transport into plastids of barley depends on the intraplastidic availability of one of its substrates, protochlorophyllide (PChlide). The precursor of POR (pPOR), synthesized from a corresponding full-length barley cDNA clone by coupling in vitro transcription and translation, is enzymatically active and converts PChlide to chlorophyllide (Chlide) in a light- and NADPH-dependent manner. Chlorophyllide formed catalytically remains tightly but noncovalently bound to the precursor protein and stabilizes a transport-incompetent conformation of pPOR. As shown by in vitro processing experiments, the chloroplast transit peptide in the Chlide-pPOR complex appears to be masked and thus is unable to physically interact with the outer plastid envelope membrane. In contrast, the chloroplast transit peptide in the naked pPOR (without its substrates and its product attached to it) and in the pPOR-substrate complexes, such as pPOR-PChlide or pPOR-PChlide-NADPH, seems to react independently of the mature region of the polypeptide, and thus is able to bind to the plastid envelope. When envelope-bound pPOR-PChlide-NADPH complexes were exposed to light during a short preincubation, the enzymatically produced Chlide slowed down the actual translocation step, giving rise to the sequential appearance of two partially processed translocation intermediates. However, ongoing translocation induced by feeding the chloroplasts δ-aminolevulinic acid, a precursor of PChlide, was able to override these two early blocks in translocation, suggesting that the plastid import machinery has a substantial capacity to denature a tightly folded, envelope-bound precursor protein. Together, our results show that pPOR with Chlide attached to it is impaired both in the ATP-dependent step of binding to a receptor protein component of the outer chloroplast envelope membrane, as well as in the PChlide-dependent step of precursor translocation.

PLASTID proteins are encoded by two different genetic systems, the nucleus and the plastid (for review see Thompson and White, 1991; Sugiura, 1992; for review). Most of the organellar proteins are coded for by nuclear genes (Tobin and Silverthorne, 1985; Thompson and White, 1991), are synthesized as larger precursor molecules in the cytosol, and must be transported posttranslationally into the plastids (Keegstra et al., 1989; Archer and Keegstra, 1990). Numerous steps have been identified that are involved in the targeting of the precursor proteins to the chloroplasts and their different suborganellar compartments. These include cytosolic synthesis of the precursors with NH_2-terminal extensions (Hightower and Ellis, 1978; Schmidt et al., 1979), referred to as the chloroplast transit peptides (see Keegstra et al., 1989; Archer and Keegstra, 1990; for a compilation of these amino acid sequences), energy-dependent binding of the precursor proteins to the outer plastid surface (Pfisterer et al., 1982; Cline et al., 1985; Olsen et al., 1989; Olsen and Keegstra, 1992), and translocation of the proteins across the outer and inner plastid envelope membranes Flügge and Hinz, 1986; Theg et al., 1989; Waegemann and Soll, 1991; Soll and Waegemann, 1992; Schnell and Blobel, 1993). During or shortly after membrane passage, the NH_2-terminal chloroplast transit peptides are cleaved off by the leader peptidase (Robinson and Ellis, 1984; Oblong and

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This article is dedicated to Professor Dr. D. Schlee, our teacher and friend.

Lamppa, 1992*a*). Before, during, and after translocation, complex protein-protein interactions between the precursor polypeptides and cytosolic (Waegemann et al., 1990; Grimm et al., 1991), membrane (Ko et al., 1992; Waegemann and Soll, 1991) and stromal factors (Payan and Cline, 1991; Breimann et al., 1992; Hartman et al., 1992; Yalofsky et al., 1992; Tsugeki and Nishimura, 1993), respectively, have been demonstrated or have at least been proposed to occur. In addition, enzymes must bind cofactors, prosthetic groups, and substrates, to attain their final conformations and activities. In the case of multimeric enzymes, protomers have to assemble into the active holocomplexes (Gatenby et al., 1988; Ellis, 1990; Hubbs and Roy, 1992; Chen and Jagendorf, 1993). Postimport routing is required for proteins that are destined for either the thylakoid membranes or the thylakoid lumen (Cline et al., 1992; Robinson et al., 1993).

In contrast to most of these different steps, little is known about the actual translocation process by which the precursor proteins cross the outer and inner plastid envelope membranes. Recent results have identified protein components both of the outer (Cornwell and Keegstra, 1987; Salomon et al., 1990; Siegenthaler and Bovet, 1993) and inner plastid envelope membranes (Li et al., 1992) that are supposed to be involved in the process of precursor translocation, and an entire transport apparatus has been postulated (Waegemann and Soll, 1991; Soll and Waegemann, 1992; Schnell and Blobel, 1993). Thus far, however, only a few studies have addressed precursor protein-specific requirements imposed by the transport machinery on the translocation process (Carillo, 1985; Takahashi et al., 1986; Lubben et al., 1987; della-Cioppa and Kishore, 1988; Fernandez and Lamppa, 1990; Cline et al., 1992; Ko and Ko, 1992; Oblong and Lamppa, 1992*b*; Pilon et al., 1992*a*,*b*; Guera et al., 1993; America et al., 1994). In particular, the influence of substrates, cofactors, or prosthetic groups on precursor protein conformation, as well as the timing and location at which these compounds become attached to the proteins, have not been investigated in detail in most cases.

We have studied the effects of protochlorophyllide (PChlide)[1], NADPH and chlorophyllide (Chlide) on the transport of the in vitro-synthesized barley precursor of the NADPH:protochlorophyllide oxidoreductase (POR) into plastids. This key enzyme of chlorophyll biosynthesis in higher plants (Griffiths, 1978; Apel et al., 1980) is encoded in the nucleus, synthesized on cytoplasmic ribosomes and subsequently translocated into the plastids, where its processing to the mature size occurs. The in vitro-synthesized pPOR is enzymatically active and forms a photoactive ternary pPOR-PChlide-NADPH complex that, upon illumination, reduces the PChlide, to Chlide. This fact has been exploited in the present work. We demonstrate that enzymatic product formation impairs both the precursor protein's binding to and translocation across the plastid envelope.

Materials and Methods

In Vitro Transcription and Translation

The *por*-specific full-length cDNA from barley, designated A7, was isolated and characterized by Schulz et al. (1989). Bluescript DNA containing the *por* insert was linearized by digestion with HindIII and used for in vitro transcription with T7 RNA polymerase according to Krieg and Melton (1984). After the incubation, nucleic acids were recovered by ethanol precipitation, and the plasmid DNA was removed by DNase digestion (Maniatis et al., 1982). The *por*-specific transcript was used to program a cell-free translation system from wheat germ as described previously (Reinbothe et al., 1990).

Enrichment of pPOR-Substrate and pPOR-Product Complexes

After terminating the translation by the addition of an excess of L-methionine (8.3 mM final concentration) and cooling of the samples on ice, the activity of the in vitro-formed pPOR was determined as follows. 50-μl assays, consisting of 25 μl of the in vitro translation mixture, 2.5 μl of isolated PChlide (15 μM final concentration) and 20 μl of assay buffer containing 100 mM Hepes-KOH, pH 7.2, 660 mM sucrose, 5 mM ATP, 5 mM $MgCl_2$, 2 mM EDTA, 17 mM L-methionine, were mixed under green safe light at 4°C. After a short preincubation to adjust the final reaction temperature to 25°C, 2.5 μl of NADPH (0.5 mM final concentration) were added to initiate the reaction. Control assays contained doubly distilled water instead of either NADPH or NADPH plus PChlide. After a 15-min dark or light incubation, the assays were loaded onto disposable 1-ml columns filled with Sephadex-G25 (Olsen et al., 1989) equilibrated with the import buffer described by della-Cioppa et al. (1986) but lacking ATP. After a dark centrifugation at 3,000 rpm in a Sorvall RC-5B centrifuge (Dupont de Nemours, Wilmington, Delaware), rotor HB4, for 1 min, the different pPOR-substrate and pPOR-product complexes were detected in the flow-through by their blue light-induced fluorescence emissions at 628 and 665 nm, respectively, measured at an excitation wavelength of 431 nm in a fluorescence spectrometer LS50 (Perkin Elmer Corp., Norwalk, CT).

Protein Transport into Isolated, Purified Barley Chloroplasts

Seedlings of barley (*Hordeum vulgare* L. cv. Carina) were germinated on moist vermiculite and grown at 23°C in the light at 30 W/m^2 (fluorescent bulbs) or in the dark for 5 d. Chloroplasts and etioplasts, respectively, from such seedlings were isolated by Percoll (Pharmacia LKB Biotechnology AB, Sweden) density gradient centrifugation (Gomez-Silva et al., 1985*a*), in combination with a second purification step on Percoll cushions (Grossman et al., 1982), as described previously (Reinbothe et al., 1990). After a final sedimentation, the plastids were resuspended in the import buffer of della-Cioppa et al. (1986) at a concentration of 0.1 mg chlorophyll/ml, determined according to Arnon (1949). Chloroplasts to be used to study the transport of pPOR across the plastid envelope membranes, including the steps of receptor binding and translocation, were first incubated with δ-aminolevulinic acid (ALA, 0.5 mM final concentration) in the dark for 15 min to raise the intraplastidic level of PChlide (Gomez-Silva et al., 1985*b*) and were subsequently kept on ice for 1 h to deplete them of ATP (Theg et al., 1988). Chloroplasts to be used for binding assays only were not fed with ALA but were depleted of ATP, as described above. After addition of ATP to a final concentration of 5 mM (della-Cioppa et al., 1986), assays composed of the in vitro-synthesized, gel-filtered pPOR, pPOR-substrate, or pPOR-product complexes, respectively, and chloroplasts containing or lacking PChlide were incubated in the dark for 15 min. Thereafter, the chloroplasts were sedimented by centrifugation and further purified through Percoll cushions as described previously. Those chloroplasts that had been used only for binding assays were diluted with a tenfold excess of a solution containing 5% (wt/vol) TCA, and their proteins were further analyzed by PAGE (see below). The plastids recovered after the import reaction with the [^{35}S]methionine-labeled pPOR were subjected to thermolysin treatment as described by Cline et al. (1984). Precursor POR molecules not bound to or sequestered by the plastids were recovered by precipitation with TCA (5% [wt/vol] final concentration) from the supernatant obtained after centrifugation of the import mixture at the end of the incubation. To measure the protease sensitivity of the chloroplast-bound pPOR and the pPOR-PChlide-NADPH complex, the protocol of Eilers et al. (1988) was used, except that intact organelles were not lysed before the addition of trypsin (10 μg/ml final concentration). For the experiment described in Fig. 7, either continuous white light (ca. 1 W m^{-2}, provided by fluorescent bulbs) or a single light pulse (provided by a Minolta 200× flash light) were used.

In Vitro Processing Assay

Wheat germ extracts containing leader peptidase activity were prepared as

1. *Abbreviations used in this paper*: ALA, δ-aminolevulinic acid; Chlide, Chlorophyllide; PChlide, protochlorophyllide; pPOR, (precursor) NADPH: protochlorophyllide oxidoreductase.

described previously (Reinbothe et al., 1990). Different amounts of the various extracts were tested for their activity to process either the gel-filtered, naked pPOR, or the pPOR contained in the various pPOR-substrate and pPOR-product complexes. The pPOR-PChlide, pPOR-PChlide-NADPH, and pPOR-Chlide complexes were formed by incubating the in vitro-synthesized pPOR with its substrates either in the dark or in the light during a 15-min preincubation in assays lacking an endogenous leader peptidase activity, followed by their separation by gel filtration to remove the excess of nonenzyme-bound substrates (see above). 25 μl of the flowthroughs containing the different pPOR-substrate and pPOR-product complexes were subsequently added to an equal volume of wheat germ extract (0.1 mg ml^{-1} protein) containing leader peptidase activity. After a 15-min incubation at 25°C in the dark, the assays were diluted with 195 μl of the import buffer (see above), followed by the addition of 80 μl of a solution containing 20% (wt/vol) TCA. Proteins were recovered by centrifugation and processed for PAGE as described previously (see above).

Miscellaneous

Protochlorophyllide was isolated from dark grown barley seedlings as described by Griffiths (1978). Similarly, Chlide was prepared from dark grown seedlings that had been exposed to light for 30 min before the isolation of the pigment. All operations were performed at 4°C under green safe light. Denaturing, SDS-containing 11–20% polyacrylamide gradients used for protein separation were prepared and run as described by Laemmli (1970). The abundances of pPOR and POR were estimated by autoradiography and densitometry.

Results

Enzymatically Produced Chlorophyllide Prevents the Protochlorophyllide-dependent Transport of pPOR into Chloroplasts

We have recently demonstrated that pPOR, synthesized in vitro from a corresponding full-length barley cDNA clone, is taken up by plastids only if the organelles contain PChlide (Reinbothe et al., 1994). This pigment can be applied either externally, leading to its internalization by the plastids, or it can be produced from ALA inside the plastids (Reinbothe et al., 1994). Enzyme-bound PChlide does not interfere with the transport of pPOR into PChlide-containing plastids (Reinbothe et al., 1994). However, enzymatic product formation, which occurred during a 15-min light preincubation of pPOR with PChlide and NADPH, impaired the subsequent transport of the [^{35}S]methionine-labeled precursor protein into chloroplasts in assays supplemented with PChlide (Fig. 1). This is seen by the obvious lack of mature POR appearing inside the chloroplasts and the constant level of pPOR remaining outside the plastids after a 15-min dark incubation (Fig. 1), confirming previous results (Reinbothe et al., 1994). In contrast, PChlide plus NADPH did not inhibit the import of pPOR into chloroplasts, if the in vitro translation assay had been preincubated in the dark (Fig. 1). In assays lacking NADPH, pPOR was imported into chloroplasts independent of whether the preincubation with PChlide had been performed in the light or in the dark (Fig. 1).

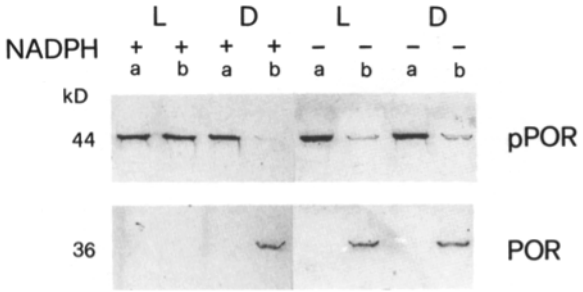

Figure 1. The effect of PChlide, PChlide plus NADPH, and enzymatically formed Chlide on transport of pPOR into chloroplasts. [^{35}S]Methionine-labeled POR precursor molecules (pPOR) were synthesized by coupled in vitro transcription/translation of a *por*-specific cDNA. After synthesis, the assays were supplemented with PChlide, and with (+) or without (−) NADPH. After a 15-min light (*L*) or dark (*D*) incubation, the assays were diluted with one fourth volume of a fourfold-concentrated import buffer (see Materials and Methods). Chloroplasts were added, and the transport reaction was performed in the dark for 15 min. Thereafter, the chloroplasts were separated from the incubation medium by centrifugation, followed by their repurification by flotation and resedimentation. After treatment of the plastids with thermolysin, their proteins were prepared and processed for PAGE (see Materials and Methods). POR precursor molecules not sequestered by the chloroplasts were recovered by precipitation with TCA from the supernatant fraction obtained after sedimentation of the chloroplasts at the end of the transport reaction. Proteins from the supernatant and chloroplast fractions were run in separate denaturing 11-20% polyacrylamide gradients, and both the radiolabeled pPOR and POR were detected by autoradiography. Lanes *a* and *b* each show proteins recovered before and after the transport reaction, respectively.

Exogenously Added Chlorophyllide Does Not Impair the Transport of pPOR into Plastids

One may speculate that freshly formed Chlide could have dissociated from the precursor protein and subsequently interfered with the transport process by interacting with a component of the transport apparatus in the outer plastid envelope. If this explanation would hold true, exogenously applied and enzymatically produced Chlide should exert similar negative effects on the PChlide-dependent transport of pPOR into plastids. To test this hypothesis, Chlide was isolated from dark grown barley seedlings that had been exposed to light for 30 min (Griffiths, 1978).

Chlorophyllide added exogenously to the assays had no effect on the PChlide-dependent transport of pPOR into chloroplasts (Fig. 2). Since PChlide and Chlide, both of which were contained in the assays, might have had different

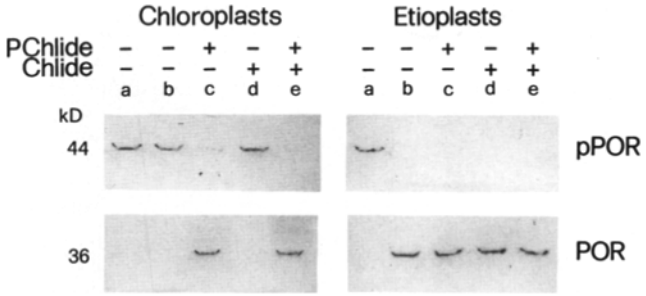

Figure 2. The effect of exogenously added Chlide on transport of pPOR into isolated plastids. Chloroplasts and etioplasts were isolated from barley seedlings that had been grown for 5 d in the light or in the dark, respectively. PChlide and Chlide were prepared from barley seedlings as described in the text. The pigments were added in the indicated combinations at a final concentration of 15 μM and 150 μM, respectively, to import assays containing the in vitro-synthesized [^{35}S]methionine-labeled pPOR and chloroplasts or etioplasts. Control assays were devoid of these two compounds. Before (lane *a* each) and after a 15-min dark incubation (lanes *b-e* each), pPOR and POR were recovered and processed for PAGE as described in Fig. 1.

binding constants and thus competed for a common component of the transport apparatus in the outer plastid envelope, we next investigated the effect of exogenously applied Chlide on pPOR transport into etioplasts. This plastid type has recently been shown to import pPOR even in the absence of exogenously added PChlide, likely due to the sufficiently high endogenous level of this pigment (Reinbothe et al., 1994). As found for chloroplasts, Chlide did not impair the transport of pPOR into etioplasts (Fig. 2), suggesting that the pigment likely does not exert its negative effect on pPOR transport by binding to a protein receptor component exposed to the outer surface of the plastid envelope.

Enzymatically Formed Chlorophyllide Remains Tightly Bound to pPOR

We next tested whether enzymatically formed Chlide might inhibit transport of pPOR into chloroplasts by remaining bound to the precursor protein and thus stabilizing a transport-incompetent protein conformation. To analyze the physical nature of this putative Chlide-pPOR interaction, assays containing pPOR, PChlide, and NADPH were exposed to light during a 15-min preincubation. Before centrifugation through columns of Sephadex-G25, a step which has recently been shown to allow the enrichment of pPOR-substrate and pPOR-product complexes (Reinbothe et al., 1994), one aliquot of the assay was supplemented with SDS to a final concentration of 0.5% (wt/vol). As demonstrated in Fig. 3, this treatment caused the complete loss of the pigment, in comparison to the untreated control recovered as a Chlide-containing pPOR complex after centrifugation. Similarly, heat denaturation led to the dissociation of the pPOR-Chlide complex (data not shown), demonstrating the noncovalent binding of Chlide to pPOR.

Chlorophyllide Formation Renders pPOR Incompetent for Binding to the Plastid Envelope Membranes

Since freshly formed Chlide remains tightly bound to the POR precursor protein, we investigated which step of the transport process, the binding of the precursor protein to or its subsequent translocation across the plastid envelope, might be impaired by the enzyme's pigment product. Chloroplasts were first incubated in the dark with ALA to cause intraplastidic PChlide accumulation (Gomez-Silva et al., 1985*b*). Controls without ALA were incubated under identical conditions with phosphate buffer alone. After the incubation, the chloroplasts were kept on ice for 1 h to deplete them of ATP (Theg et al., 1989). Energy sources that were contained in the mixture used for in vitro translation were removed by gel filtration on Sephadex-G25 (Olsen et al., 1989) equilibrated in the import buffer described by della-Cioppa et al. (1986) but devoid of ATP. POR precursor molecules eluting in the flowthrough during gel filtration were added to chloroplasts containing or lacking the PChlide produced by ALA feeding. After a 15-min dark incubation on ice, the chloroplasts were sedimented by centrifugation and repurified by flotation on Percoll cushions, followed by resedimentation.

As demonstrated in Fig. 4, the "naked" pPOR, i.e., the polypeptide not bearing either its substrates or products, was imported into chloroplasts containing PChlide produced by ALA feeding in assays supplemented with ATP. If pPOR was incubated with chloroplasts lacking PChlide, the precursor protein was still able to bind the plastid envelope but it was not imported into the organelle. This is evident from the quantitative shift of pPOR from the supernatant to the plastid fraction (Fig. 4, lane *b*). In the absence of ATP, the precursor protein level remained constant in the supernatant fraction, and no pPOR cosedimented with the chloroplasts (data not shown). Similar results were obtained for PChlide-containing chloroplasts in assays lacking ATP (data not shown).

To study the chloroplast-binding properties of the different binary and ternary pPOR-substrate or pPOR-product complexes, pPOR-PChlide, pPOR-PChlide-NADPH, and pPOR-Chlide(-NADP) complexes were enriched by centrifugation through Sephadex-G25 columns (see Materials and Methods). As demonstrated in Fig. 4, the two pPOR-substrate complexes differed markedly from the pPOR-product complex with respect to binding to the chloroplast envelope. In neither case did substrate attachment have an effect on the subsequent ATP-dependent binding of pPOR to the chloroplasts (Fig. 4, lane *b* each). If the chloroplasts contained PChlide, pPOR was taken up and processed to its mature size independently of whether the precursor protein had been preincubated with or without its substrates (Fig. 4, lane *c*

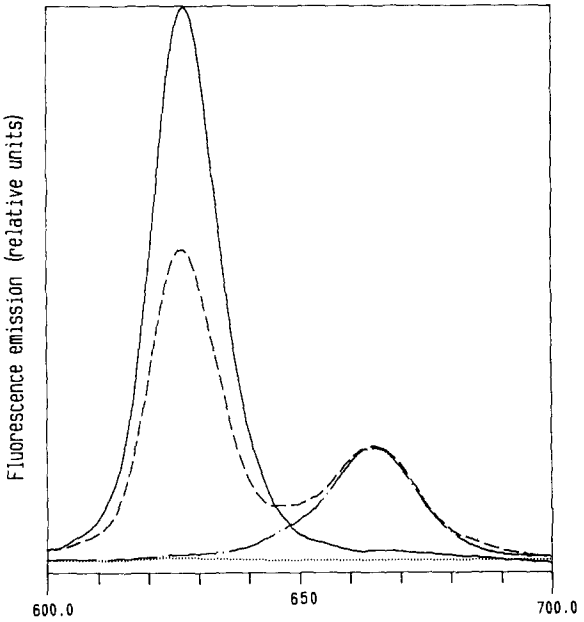

Figure 3. Enzymatic Chlide formation by pPOR. The in vitro-synthesized pPOR supplemented with its two substrates, PChlide, and NADPH, as described in Fig. 1, and the assays were immediately divided into four equal parts. One aliquot was immediately used for fluorescence spectroscopy, while the other three parts were incubated in the light for 15 min. Thereafter, one of the illuminated samples was used to determine its fluorescence properties. Another one of the illuminated samples was treated with SDS (0.5% final concentration), while the remaining third sample was left untreated. The latter two samples were then subjected to gel filtration on Sephadex-G25, equilibrated with the import buffer described in the text. The different curves show the fluorescence emission spectra of the nongel-filtered assays before (—) and after the light incubation (– – –), and of the gel-filtered, illuminated assays after pretreatment with (·····) or without SDS (–·–). PChlide and Chlide have maxima of fluorescence emission at 628 nm and 665 nm, respectively, at an excitation wavelength of 431 nm. The spectra were normalized at 665 nm.

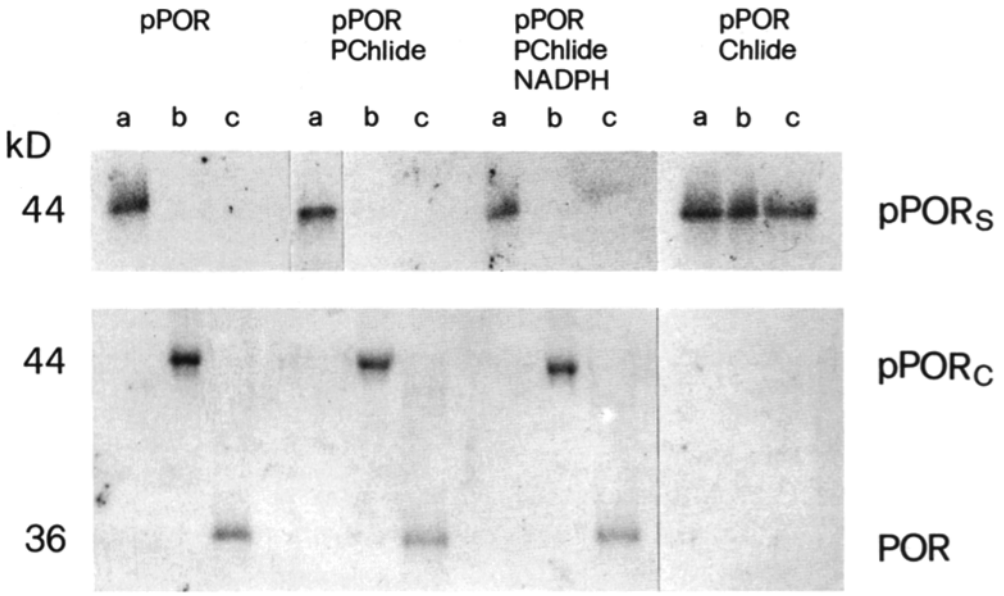

Figure 4. Chloroplast receptor-binding properties of pPOR, pPOR-substrate and pPOR-product complexes. Chloroplasts, isolated from light-grown seedlings as described in Fig. 1, were incubated with ALA (0.5 mM final concentration) in the dark for 15 min to cause intraplastidic PChlide formation. Control assays contained phosphate buffer instead of ALA. Thereafter, the chloroplasts were kept on ice for 1 h. pPOR was synthesized by in vitro translation and was subsequently supplemented with either PChlide or PChlide plus NADPH; controls were left unsupplemented. Assays containing the ternary pPOR-PChlide-NADPH complex were incubated in the dark or in the light for 15 min, while all other assays were kept in the dark. All of the different assays were then subjected to gel filtration as described in Fig. 3 and were added to chloroplasts lacking or containing the PChlide produced by ALA feeding in assays supplemented with ATP (5 mM final concentration). After a 15-min dark incubation on ice, proteins were recovered from the supernatant and the chloroplast fractions, obtained after centrifugation, as described in Fig. 1, with the modification that those plastids that had been used only for the binding of pPOR were not treated with thermolysin (lane *b* each). The autoradiograms show free ($pPOR_s$, lane *a*) and chloroplast-bound POR precursor molecules ($pPOR_c$, lane *b*), as well as the mature, thermolysin-resistant POR (lane *c*), each before (lane *a*) and after the incubation with chloroplasts lacking or containing PChlide (lanes *b* and *c*, respectively).

each). In contrast, enzyme-bound Chlide, produced during a light preincubation, completely blocked the binding of pPOR to the chloroplasts and thus abolished the whole transport process (Fig. 4).

Based on the latter result, we assumed that the pPOR-Chlide complex might have adopted a compact overall conformation in which the chloroplast transit peptide was no longer able to physically interact with the outer plastid envelope. To demonstrate this, an in vitro processing experiment was performed. Because chloroplasts have previously been shown to contain proteases that rapidly degrade POR after

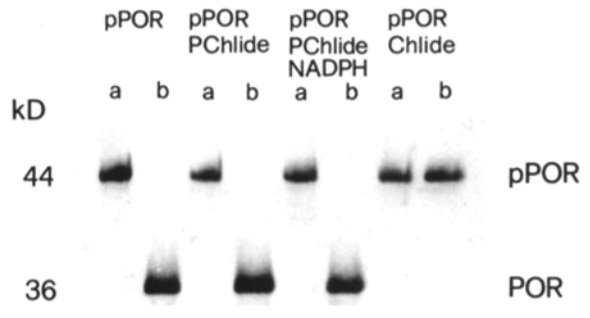

Figure 5. In vitro processing of pPOR, pPOR-PChlide, pPOR-PChlide-NADPH, and pPOR-Chlide complexes. The different pPOR-substrate and pPOR-product complexes were produced as described in Fig. 3 and were subsequently incubated with a wheat germ protein extract containing leader peptidase activity. Either before (lane *a* each) or after a 15-min incubation in the dark (lane *b* each), proteins contained in the various assays were recovered and processed for PAGE as described in Fig. 1.

Chlide formation in the light (Kay and Griffiths, 1983; Häuser et al., 1984; Forreiter et al., 1990), we had to look for another source of leader peptidase activity. Taking previous findings into account that artificial processing of plastid precursor proteins had occasionally been observed in wheat germ extracts (Pfisterer et al., 1982; Reinbothe et al., 1990), several different batches of wheat germ were tested for leader peptidase activity arising from proplastids broken during the preparation of the protein-synthesizing system. Out of 23 wheat germ batches analyzed, the most active preparation was used to treat the naked pPOR and the various pPOR-substrate and pPOR-product complexes with the leader peptidase contained in the protein extract. As shown in Fig. 5, the naked pPOR as well as the pPOR-substrate complexes were completely processed into the mature POR, while no processing occurred in assays containing the pPOR-Chlide complex.

pPOR Loaded with its Substrates Retains a Folded Conformation Even after Binding to the Plastid Envelope

We next addressed the question of whether pPOR with PChlide plus NADPH bound to it, may become unfolded upon binding to the plastid envelope. Taking previous findings of Guera et al. (1993) into account, showing a strong unfolding activity to be present in the plastid envelope, we speculated that the apparent lack of PChlide and NADPH to inhibit the transport process might be due to their release from the different pPOR-substrate complexes upon binding of pPOR to the outer envelope membrane. To study this, chloroplasts lacking PChlide were used for the binding reac-

tion with the pPOR-PChlide-NADPH complex; as a control, the naked pPOR was included. After binding, the chloroplasts were treated with trypsin. As demonstrated in Fig. 6 *A*, such postbinding protease treatment caused the rapid degradation of the naked, chloroplast-bound POR precursor protein. In contrast, the attachment of PChlide and NADPH to pPOR before its binding to the chloroplasts correlated with a stabilization of the plastid-bound precursor protein towards trypsin treatment (Fig. 6 *B*).

Nascent Chlorophyllide Formed by Envelope-bound pPOR-PChlide-NADPH Complexes Inhibits Translocation of pPOR

When ternary pPOR-PChlide-NADPH complexes were bound to PChlide-free chloroplasts during a dark preincubation, their subsequent translocation driven by ALA feeding was impaired if performed in the light (Fig. 7 *A*). Two distinct chloroplast-bound intermediate size pPOR derivatives (iPOR) with molecular masses of 41 and 39 kD, respectively, appeared transiently (Fig. 7 *A*). The accumulation of the 39-kD iPOR was delayed, suggesting that it was formed from those 41-kD protein molecules that were able to override the early block in translocation. Because the chloroplasts were not treated with thermolysin, both iPORs must represent partially processed and thus truncated pPOR derivatives. One can therefore conclude that the stromal peptidase must have been able to recognize and cleave the partially accessible chloroplast transit peptide, exposed to the stroma by the arrest of translocation, at two specific sites. Chloroplast-bound ternary pPOR-PChlide-NADPH complexes that had not been exposed to light were sequestered by the chloroplasts without an apparent accumulation of the two pPOR derivatives intermediates (Fig. 7 *B*).

The obvious lack of mature POR cosedimenting with the chloroplasts after the light incubation might be explained in two ways. First, enzymatic Chlide formation might have caused the release of a significant portion of the envelope-bound pPOR molecules to the cytosolic side of the chloroplasts. This explanation is not very likely, however, since no pPOR could be found in the supernatant fraction recovered at the end of the light incubation (Fig. 7 *C*). Alternatively, imported and processed POR molecules might have been degraded within the chloroplasts during illumination. To address this question, two different experiments were performed. First, chloroplast-bound pPOR-PChlide-NADPH complexes were exposed to a brief light pulse, while their subsequent transport into the plastids was performed in complete darkness. As shown in Fig. 7 *D*, such a brief light incubation did cause the accumulation of the two previously identified pPOR derivatives (cf. Fig. 7 *A*) as well as the delayed appearance of the mature 36-kD POR, all cosedimenting with the chloroplasts.

Second, the stability of imported and processed POR molecules was investigated by incubating the PChlide-containing chloroplasts after the transport reaction either in the light or in the dark for an additional period. As demonstrated in Fig. 8, such a postimport incubation caused the rapid loss of POR in the light, but did not affect the level of POR in the dark.

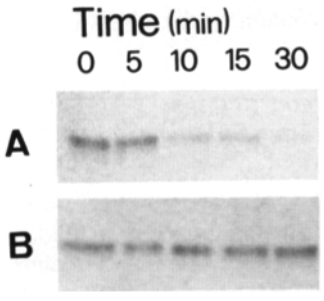

Figure 6. Attempts to demonstrate differences in conformation of the chloroplast-bound pPOR (*A*) and pPOR-PChlide-NADPH complex (*B*). The binding reaction of the radiolabeled and gel-filtered pPOR or pPOR-PChlide-NADPH complex to PChlide-free chloroplasts was performed in the dark as described in Fig. 4. After resedimentation and repurification, the chloroplasts were treated with trypsin as described in the text, and their proteins were subsequently processed for PAGE as described in Fig. 1. The autoradiograms show time courses of pPOR abundance depending on the duration of protease treatment.

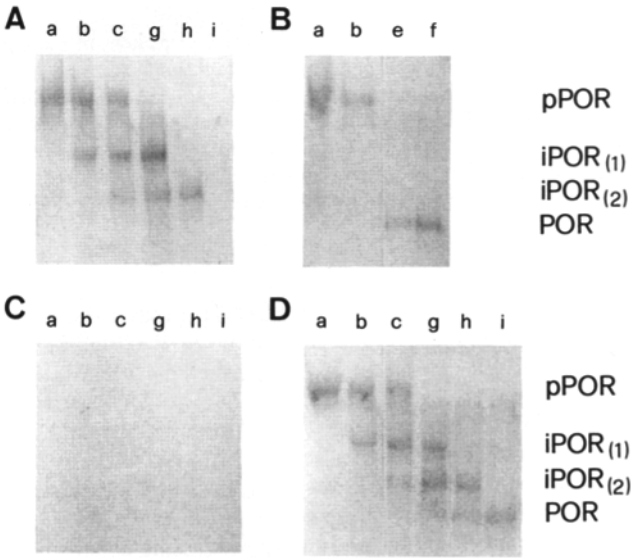

Figure 7. Impairment by light of translocation of the chloroplast-bound pPOR bearing PChlide plus NADPH. The binding of PChlide plus NADPH to pPOR synthesized in vitro was performed in the dark as described in Fig. 3. After gel filtration, the pPOR-PChlide-NADPH complex was bound in the dark to PChlide-free chloroplasts (*A*, *B*, and *D*, lane *a* each), as described in Fig. 4. Chloroplasts bearing the ternary pPOR-substrate complex were subsequently fed with ALA either in continuous light (*A* and *C*) or in complete darkness (*B* and *D*), for 0 (*a*), 1 (*b*), 1.5 (*c*), 3 (*e*), 5 (*f*), 10 (*g*), 15 (*h*), and 30 min (*i*), respectively. At the beginning of the incubation, one part of the chloroplasts to be incubated in the dark was subjected to a short light pulse (*D*). After centrifugation of the assays, proteins were recovered by precipitation with TCA from the supernatant fractions of those assays that had been incubated in continuous light (*C*). Chloroplasts were repurified by flotation and sedimentation, and their proteins were recovered by sonication and TCA precipitation (cf. Fig. 1) except that the step of thermolysin treatment was omitted. Proteins present in the supernatant and chloroplast fractions, respectively, were separated in denaturing polyacrylamide gels. The autoradiograms show the radiolabeled chloroplast-bound 44-kD POR precursor protein ($pPOR_c$), two translocation intermediates (iPOR) as well as the mature 36-kD POR.

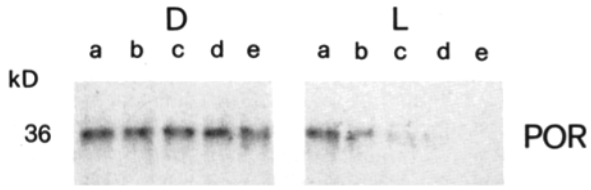

Figure 8. Postimport stability of POR in the dark (*D*) or in the light (*L*). The naked, gel-filtered pPOR was imported into PChlide-containing chloroplasts in the dark in assays supplemented with ATP as described in Fig. 4. Thereafter, one part of the chloroplasts was kept in the dark, while another part was transferred into the light. After 0 (*a*), 2 (*b*), 5 (*c*), 10 (*d*), and 15 min (*e*), respectively, the abundance of the mature POR was determined by autoradiography.

Discussion

Tight Folding, Induced by Enzymatic Chlorophyllide Formation, Impairs Both the Receptor Binding Function as well as Translocation Competence of pPOR

Unfolding seems to be a general prerequisite for the translocation of proteins across biological membranes (Eilers and Schatz, 1986, 1988; Verner and Schatz, 1988; Neupert et al., 1990; Driessen, 1992; Rapoport, 1992; Simon et al., 1992). To our knowledge, there are only a few examples reporting the transfer of folded proteins across membranes, such as transport of bacterial pullulanase (Pugsley, 1992) or α-lytic protease (Fujishige et al., 1992) across the plasma membrane of *Escherichia coli*. Thus far, it is still undetermined, however, whether the different forces proposed to be required for the actual translocation process, such as cotranslational polypeptide chain elongation, ATP hydrolysis, or electrochemical gradients (summarized in Neupert et al., 1990; Glick and Schatz, 1991; Driessen, 1992; Rapoport, 1992; Sanders and Schekman, 1992; Simon et al. 1992), may be involved in driving either the unfolding reaction or the movement of the different precursor polypeptides during membrane passage, or whether they even promote both.

In the particular case of pPOR, we propose that intraplastidic PChlide, formed from ALA, might drive the translocation of the precursor protein by causing or supporting its refolding at the stromal side of the plastid envelope. Supporting this view, PChlide synthesis has previously been shown to occur in the plastid envelope (Joyard et al., 1990, 1991). This site of pigment biosynthesis would ensure that freshly formed PChlide is ready to interact with the pPOR. Because pPOR has to bind to the plastid envelope before its transport, the first interaction between PChlide and the precursor protein thus can be expected to take place in the NH_2-terminal transit peptide. This interaction would set in motion the actual translocation process. After the transit peptide has passed the plastid envelope, this process might be driven by binding of PChlide to those residues in the mature region that have previously been demonstrated to be required for enzyme catalysis, such as the cysteine residues (Apel et al., 1980). Thus, at least two or even more PChlide-binding sites might be predicted to exist per pPOR molecule, one being present in the transit peptide and the other one (or two) in the mature region of the polypeptide. Confirming this prediction, previous experiments had demonstrated that each POR molecule was bound to two to three PChlide molecules (Apel et al., 1980).

As a consequence of the postulated (precursor) enzyme-substrate interaction, any competing reaction outside the plastids, such as product formation, would impair unfolding of pPOR during membrane passage and thus should inhibit the transport process. In accordance with this prediction, enzymatic Chlide formation did impair the transport of pPOR into plastids. The pigment product of catalysis remained tightly bound to pPOR and caused the establishment of a transport-incompetent pPOR conformation in which the chloroplast transit peptide was no longer able to bind to the outer plastid envelope membrane. When chloroplast-bound ternary pPOR-PChlide-NADPH complexes were exposed to a short light pulse, followed by their incubation in the dark, or were kept in continuous light, two distinct chloroplast-bound intermediate size pPOR derivatives appeared in both cases. Presumably, the catalytically formed Chlide had slowed down the translocation of pPOR across the plastid envelope membranes. However, the ongoing translocation process appeared to be able to override the early blocks in pPOR transport, as seen by the sequential accumulation of the first and second pPOR derivatives.

pPOR Enters the Chloroplasts Likely in an Unfolded Conformation

If pPOR with either its substrates or product(s) attached to it would be transferred across the plastid envelope in a folded conformation, then one could expect the PChlide and Chlide should exert similar negative effects on the transport process. This was obviously not the case, however. Only Chlide-containing but not PChlide-containing chloroplast-bound pPOR complexes were impaired in translocation across the plastid envelope. On the other hand, it would be difficult to imagine how intraplastidic PChlide could drive the translocation of pPOR if there were no free binding sites in the (folded) precursor protein ready to interact with the pigment. Finally, imported POR-Chlide complexes that had been formed during a short light incubation should have been similarly proteolytically degraded in the dark as POR-PChlide-NADPH complexes that, either during or after their translocation into the plastids in the light, had continuously converted their PChlide into Chlide. Whereas the obvious lack of mature POR in chloroplasts that had been continuously illuminated might be explained by translocation of the pPOR-PChlide-NADPH complex plus rapid proteolytic degradation of the resulting pPOR-Chlide complex, the detection of imported and processed POR molecules in the flash-illuminated chloroplasts appears to disprove the possibility that the tightly folded pPOR-Chlide complex entered the plastids. It is therefore more likely that intraplastidic PChlide binding set in motion the actual transfer of the precursor protein into the chloroplasts, thereby displacing substrates and products that were originally bound to from the precursor protein. Experiments to ultimately demonstrate this unfolding/refolding mechanism are difficult to be performed, because the lipophilic Chlide has a high affinity for and thus would, once released from the precursor protein

during translocation, likely be trapped within the lipid bilayers of the plastid envelope membranes. Presumably for a similar reason, experiments to show that ^{14}C-labeled PChlide dissociates from the chloroplast-bound pPOR-^{14}C-PChlide-NADPH complex during translocation were inconclusive thus far.

Although intraplastidic PChlide was sufficient to drive the translocation process, the pigment could not stabilize the imported, mature POR against proteolytic degradation in the light. Most probably, the imported and processed POR that had rebound PChlide and NADPH at the stromal side of the plastid envelope converted PChlide into Chlide and simultaneously became susceptible toward proteolytic degradation. In this respect, the results of the present study extend previous findings which had shown that POR, during catalysis in the light, is rapidly inactivated and subsequently degraded (Kay and Griffiths, 1983; Häuser et al., 1984; Forreiter et al., 1990). In contrast, POR was found to be protease-resistant in the dark in the presence of PChlide (and/or NADPH) (Kay and Griffiths, 1983; Häuser et al., 1984).

Protein Transport Across Mitochondrial and Other Cellular Membranes Reveals Precursor Protein-Specific Differences in Transport Competence

The results of the present paper can be compared and contrasted with those reported for other proteins destined to cross biological membranes. Ionic and hydrophobic interactions, that caused tight folding of acidic fibroblast growth factor fused to diphteria toxin (Wiedlocha et al., 1992) or oligomerization of light harvesting chlorophyll *a/b*-binding proteins expressed in *E. coli* (Oblong and Lamppa, 1992*b*), have been demonstrated to interfere with the translocation of these reporter proteins across the cellular and chloroplast envelope membranes, respectively. High affinity binding of substrate analogues, such as *N*-(phosphonomethyl)glycine (glyphosate) to binary 5-*enol*pyruvyl-shikimate-3-phosphate synthase-shikimate-3-phosphate complexes or methotrexate to cytosolic dihydrofolate reductase used as a reporter protein, similarly inhibited subsequent transport of the precursor proteins into chloroplasts (della-Cioppa and Kishore, 1988) and mitochondria (Eilers and Schatz, 1986; Eilers et al., 1988), respectively. In the latter case, the dihydrofolic acid analogue strongly reduced the targeting function of a yeast mitochondrial cytochrome oxidase subunit IV presequence (Verner and Lemire, 1989). In contrast, methotrexate binding to a similar chimeric protein, differing from the previous one only in the length of the mitochondrial presequence, did not significantly influence the binding function but diminished the translocation competence of the reporter protein (Eilers and Schatz, 1986). Similar to these in vitro studies, methotrexate caused an arrest of in vivo translocation into the intermembrane space of mitochondria of a chimeric protein comprising the NH_2-terminal third of the precursor of cytochrome b_2 fused to the dihydrofolate reductase domain (Wienhues et al., 1991). Artificially induced unfolding of the precursor proteins by either denaturation with urea or destabilization due to engineered point mutations restored the transport competence of the chimeric proteins (Eilers et al., 1988; Vestweber and Schatz, 1988; Verner and Lemire, 1989). In neither of the cited cases, however, did the translocation process itself appear to be able to drive unfolding of the dihydrofolate reductase domain if methotrexate was bound to it. Thus, the transport apparatus in the outer and inner envelope membranes of mitochondria seems to have only a limited capacity to unfold authentic and artificial precursor proteins (Pfanner and Neupert, 1990; Glick and Schatz, 1991; Pfanner et al., 1992).

In contrast, the transport machinery of chloroplasts appears to have a substantial capacity to denature tightly folded precursor proteins. Guera et al. (1993) proposed a strong unfolding activity to reside in the plastid envelope that catalyzed unfolding of two methotrexate-complexed chimeric precursor proteins, consisting of the transit sequences of ferredoxin of pea or of plastocyanin of *Silene pratensis* fused to the dihydrofolate reductase domain of mouse, upon their binding to the plastids. However, this unfolding seemed to be a rather passive process and was likely not due to a specific interaction between the precursor proteins and the transport machinery, because methotrexate was found to have a high affinity to acidic membrane lipids of the plastid envelope (America et al., 1994). In contrast to these findings, the results of the present paper for the first time provide evidence that unfolding of a tightly folded authentic plastid precursor protein, such as pPOR with Chlide bound to it, is an active process that occurs not before but during translocation.

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