The importance of the C-terminal region and Cys residues for the membrane association of the NADPH:protochlorophyllide oxidoreductase in pea

Henrik Aronsson[a],*, Christer Sundqvist[a], Michael P. Timko[b], Clas Dahlin[a,1]

[a]*Department of Plant Physiology, Göteborg University, P.O. Box 461, SE-405 30 Göteborg, Sweden*
[b]*Department of Biology, University of Virginia, Charlottesville, VA 22901, USA*

Abstract In vitro chloroplast import reactions and thylakoid association reactions have been performed with a series of C-terminal deletions and Cys-to-Ser substitution mutants of the pea NADPH:protochlorophyllide oxidoreductase (POR; EC 1.6.99). C-terminal deletions of the precursor POR (Δ362–400, Δ338–400, Δ315–400 and Δ300–400) were efficiently translocated across the chloroplast envelope. However, except the Δ396–400 mutant, no C-terminal deletion mutants or Cys-to-Ser substitution (Cys119, Cys281 and Cys309) mutants resisted posttreatment with thermolysin after the thylakoid association reactions. This suggests that these mutants were unable to properly associate to the thylakoids due to changes of the protein conformation of POR. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Membrane association; Chloroplast; NADPH:protochlorophyllide oxidoreductase mutant; Translocation

1. Introduction

The NADPH:protochlorophyllide oxidoreductase (POR) catalyzes the reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide). This is done by establishing a catalytically active ternary complex between POR and NADPH as the hydrogen donor and the pigment Pchlide as the substrate [1]. Light will then transform Pchlide to Chlide during oxidation of NADPH. In angiosperms, POR is an obligate enzyme in the chlorophyll biosynthesis.

POR is nucleus-encoded, synthesized on cytosolic ribosomes and post-translationally transported into the plastids

*Corresponding author. Fax: (46)-31-773 26 26.
E-mail addresses: har@fysbot.gu.se (Henrik Aronsson),
csu@fysbot.gu.se (Christer Sundqvist), mpt9g@unix.mail.virginia.edu
(Michael P. Timko), clas.dahlin@set.hh.se (Clas Dahlin).

[1] Present address: School of Business and Engineering, Halmstad University, P.O. Box 823, SE-301 18 Halmstad, Sweden.

Abbreviations: Δ300–400, C-terminal deletion from amino acid 300 to 400; Δ-TP, in vitro translated transit peptide-free POR protein; Cys119, Cys-to-Ser substitution; 6-His-tag, hexahistidine-tag; PIRAC, protein import-related anion channel; POR, NADPH:protochlorophyllide oxidoreductase; pPOR, precursor POR; Tic, translocon at the inner envelope membrane of the chloroplast

where it accumulates in the inner membranes of etioplasts [2] and chloroplasts [3,4]. The pea precursor POR (pPOR) contains 400 amino acids whereas the mature product is 64 amino acids shorter and contains amino acids 65–400, giving a molecular mass of approximately 36 kDa [5]. No membrane-spanning regions have been detected in POR [6], which suggests that POR is located on the stromal side of the thylakoid. Still, the major part of POR resists protease treatment when physiologically associated with the thylakoid [4].

For transport across the envelope into the plastid, all nuclear-encoded plastid proteins known, including POR [7], use the same general transport pathway characterized by the translocons at the outer and inner envelope membrane of the chloroplast, Toc and Tic.

During or shortly after the entry into the stroma, the transit peptide is cleaved off from the precursor proteins and the mature protein is destined to its functional site in the plastid [8,9]. Import, processing and membrane association of pea POR are equally efficient in chloroplasts and etioplasts, suggesting that neither Pchlide nor other etioplast specific components are involved in the import of pea POR [4,10].

Routing and association of POR with prolamellar bodies, prothylakoids and thylakoids (plastid inner membranes) require NADPH and are stimulated by ATP [4,11], but do not strictly follow any of the four distinct thylakoid targeting pathways [8,12]. The exact role for NADPH and ATP in the membrane association process is not known but it has been suggested that one or more of the three Cys residues, contained in the mature part of the protein, are involved in catalysis, Cys281, or cofactor (NADPH) binding, Cys119 [13,14]. Substitution of Cys119 to Ser119 of POR decreases but does not eliminate POR activity whereas substitution of Cys281 to Ser281 completely eliminates enzyme activity of POR (Karginova, O., Lebedev, N. and Timko, M.P., in preparation). Substitution of Cys308 to Ser308 in the C-terminal region of POR substantially decreases POR function. However, the function of Cys308 is not known.

During transport of preproteins from the cytosol to the thylakoids, the C-terminal region may be of special importance [15,16]. A protein import-related anion channel (PIRAC) has been identified in the inner envelope membrane [17]. This channel is either a Tic component or intimately associated with one or more components of the Tic complex that upon precursor binding switches from being an anion transporter to translocate proteins into stroma. PIRAC interacts with the C-terminal region of the precursor protein dur-

ing translocation [16]. The C-terminus has also proven to be essential for several nucleus-encoded thylakoid proteins for either *trans*-thylakoid transport [15,18] or insertion into the membrane [15,19]. The C-terminus of POR might be important in either interaction with the substrate, Pchlide, and/or membrane association.

This has prompted us to focus on the role of the C-terminal part of POR and in addition, on the role of the three conserved Cys amino acid residues for the thylakoid association reaction.

2. Materials and methods

2.1. Isolation of intact chloroplasts and thylakoids

Seeds of pea (*Pisum sativum* L. cv. Kelvedon Wonder) were sown in soil overlaid with vermiculite and grown in the greenhouse 8–10 days prior to chloroplast isolation. Chloroplasts were isolated from the seedlings by homogenization with grinding buffer (50 mM HEPES, 0.33 M sorbitol, 2 mM Na$_2$EDTA, 3 mM ascorbic acid, 1 mM MgCl$_2$, 1 mM MnCl$_2$, pH 7.5 KOH and 1 g/l bovine serum albumin), filtered through Miracloth and cheesecloth. The chloroplasts were isolated using a linear Percoll density gradient and further purified by differential centrifugation and washing steps [20]. The chloroplasts were resuspended in import buffer (0.33 M sorbitol, 50 mM HEPES, pH 8.0 KOH) at 1 mg chloroplasts/ml. The chloroplast concentration of the purified plastids was measured spectroscopically in 80% acetone according to [21]. To obtain lysates, later on used for thylakoid association reactions, plastids were incubated in 10 mM HEPES, pH 8.0 KOH, 10 mM MgCl$_2$ at 1 mg chloroplasts/ml and left on ice for 5 min. Lysis was confirmed by overlaying lysed chloroplasts on a 35% Percoll cushion and centrifuging at $2200 \times g$ for 8 min.

2.2. Protein expression of mutant strains

Mutations replacing Cys with Ser in pea POR were created by site-directed mutagenesis using Altered Sites II in vitro Mutagenesis System (Promega) and the following mutagenic oligonucleotides: for Cys119 5′-ATTATGGCTAGCAGGGACT-3′, for Cys281 5′-AG-CAAAGTCAGTAATATGC-3′ and for Cys308 5′-TACCCTGG-TAGCATTGCCA-3′. A *Bam*HI/*Pst*I fragment from pHM51 [1] encoding the mature pea POR was cloned into the pAlter-1 mutagenesis vector. The mutations were confirmed by sequencing the recombinant *por* genes.

POR C-terminal mutants were created by cloning PCR products, amplified from pBluescript (Stratagene) containing pea POR cDNA [5], into pET-23d vector (Novagen), using *Nco*I and *Xho*I restriction sites. The following oligonucleotides were used for amplification: N-terminus for mature POR proteins 5′-CTAGACCATGGCTCT-TCAAACTGCTT-3′ and transit peptide-free POR proteins 5′-TC-ATACGGATCCATGGAGACAGCGGCACCG-3′. C-terminus for the Δ362–400 mutant 5′-CCTTGCTCGAGACACCAGATTTTGT-TAG-3′, for the Δ338–400 5′-CCTTGCTCGAGGTAGCCTTTGGT-TATGTAC-3′, for the Δ315–400 mutant 5′-CCAACCTCGAG-CAAACCTGTTGTAGCAATG-3′ and for the Δ300–400 mutant 5′-CCTTGCTCGAGTATTCCAGTTTCTTCATG-3′. The Δ396–400 protein was achieved by linearization with *Hin*dIII of transit peptide-free POR from pAlter lpcr plasmids. A schematic diagram of the mutants is shown in Fig. 1A.

The pET-23d plasmid and the pAlter lpcr plasmid were linearized with *Pst*I and they were all extracted with standard phenol/chloroform methods. Transcription and translation were performed in T$_N$T Wheat Germ Extract System (Promega) using T7 RNA polymerase and radiolabelled [^{3}H]leucine. Before use, translation mixtures were adjusted to 30 mM unlabelled leucine. The pET9-His-POR plasmid containing cyanobacterium *Synechocystis* POR, with an N-terminal hexahistidine-tag (6-His-tag), was expressed and purified according to [14]. The protein concentration of the *Synechocystis* POR was 1.1 mg/ml.

2.3. Plastid import reaction

All steps, except for the import reaction, were run at 4°C according to [20]. A typical import reaction mixture consisted of plastids (50×10^6), radiolabelled precursor protein, MgATP (final concentra-

tion 10 mM) and import buffer to a final volume of 100 μl. The samples were incubated in light, 100 μmol/m^2 s, at 25°C for 15 min with gentle mixing of the samples every fifth minute. After import, each sample was divided and treated with or without thermolysin (final concentration 0.2 mg/ml). The thermolysin reaction was terminated after 30 min with EDTA (final concentration 5 mM) and the samples were layered on a 35% Percoll cushion and centrifuged at $2200 \times g$ for 8 min to obtain intact plastids as a pellet. The pellet was resuspended in 25 mM EDTA. Samples that were not treated with protease went through the same procedure except that import buffer was added instead of thermolysin.

2.4. Thylakoid association

All steps, except for the association reaction, were run at 4°C according to [22]. A typical association reaction mixture consisted of radiolabelled in vitro translated transit peptide-free POR protein (Δ-TP), chloroplast lysates corresponding to 50 μg chloroplasts, MgATP (final concentration 10 mM), NADPH (final concentration 2 mM) and import buffer to a final volume of 150 μl. When using *Synechocystis* POR non-radiolabelled protein was used. The samples were incubated in light, 100 μmol/m^2 s, at 25°C for 30 min. After the association reaction each sample was divided and treated with or without thermolysin (final concentration 0.2 mg/ml). The thermolysin reaction was terminated after 30 min with EDTA (final concentration 5 mM). The thylakoids were recovered by centrifugation at $2200 \times g$ for 8 min. The pellet was resuspended in 25 mM EDTA.

2.5. Protein analysis

Proteins were separated by 12.5% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) using the buffer system of Laemmli [23]. After electrophoresis, gels were prepared for fluorography or immunodetection by Western blot analyses. Proteins were blotted to nitrocellulose membrane and were incubated with mouse antibody raised against the 6-His-tag (Clontech) or with rabbit antibody raised against wheat POR [24]. The nitrocellulose membrane was further incubated with an alkaline phosphate-conjugated goat anti-mouse IgG for detection of 6-His-tagged proteins or goat anti-rabbit IgG for detection of POR. Etioplast inner membranes (EPIMs) were used as control. EPIMs were purified according to [25].
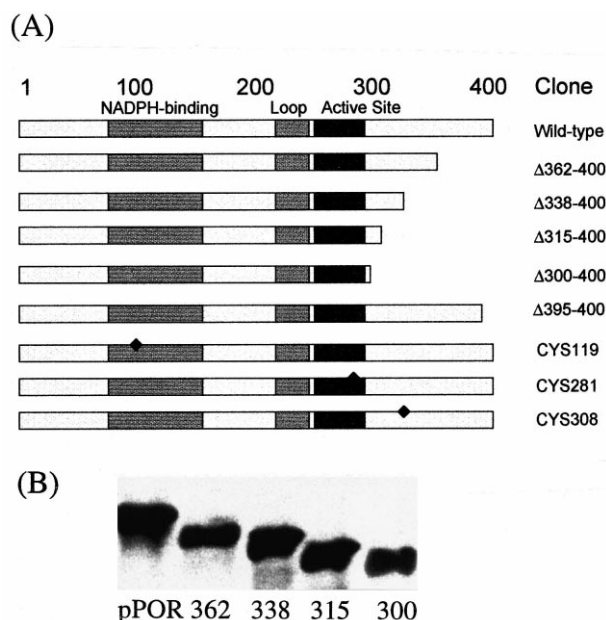

Fig. 1. A: A schematic diagram of the wild-type, the C-terminal deletion mutants and the positions of Cys-to-Ser substitution mutants of POR. The NADPH binding site, the 35 residue loop (loop) and the active site are indicated within the POR amino acid sequence. B: Fluorogram showing in vitro translated, radiolabelled wild-type and C-deleted pPOR proteins analyzed on 12.5% SDS–PAGE. pPOR, wild-type pPOR; 362, Δ362–400; 338, Δ338–400; 315, Δ315–400; 300, Δ300–400.

3. Results

3.1. Import and membrane association of C-terminal deletion mutants of POR

To determine the possible role of the C-terminal part of POR for transport across the envelope, import was performed with radiolabelled, multiple C-terminal deletions of pea pPOR into purified pea chloroplasts (Fig. 1). In the presence of 10 mM ATP, all C-terminal deletion mutants became attached to the outer envelope membrane and were further translocated over the envelope. This was shown with thermolysin treatment of repurified chloroplasts, a treatment that degraded surface-exposed precursor proteins, leaving only imported, processed POR proteins intact (Fig. 2). That is, all C-terminal deleted pPOR, in the amino acid range 300–400, was normally translocated across the envelope, just as the wild-type pPOR.

Transport across the envelope is only part of the translocation pathway of POR, since the protein has to be routed to its final suborganellar compartment, the plastid inner membranes [26]. Membrane association is regulated by the mature part of the protein [4] with a strong requirement for an intact C-terminus. This was demonstrated in thylakoid association reactions with Δ-TP and C-terminal deletion mutant proteins of Δ-TP in thylakoid association assays. The Δ-TP instead of pPOR was used for the thylakoid association reaction since the transit peptide is not necessary for the association [4]. The Δ-TP protein and all of the deletion mutants were attached to the thylakoid prior to the thermolysin post-treatment (Fig. 3A,B). However, only the Δ-TP protein and the PORΔ396–400 mutant resisted protease post-treatment, as indicated by the 34 kDa protease-protected band on the fluorographs (Fig. 3A,B). Consequently, only Δ-TP and PORΔ396–400 were associated in a physiologically proper way with the thylakoids.

The importance of the C-terminus for membrane attachment indicates that this part of the protein may function as a membrane anchor for POR. If so, it could be expected that the C-terminus is protected from the action of exogenous proteases when properly associated to the membrane. In a Western blot, POR antibodies did not detect radiolabelled pea POR proteins, with an N-terminal 6-His-tag, due to low amounts of proteins (data not shown). Instead this was investigated by using over-expressed Synechocystis POR, with an N-terminal 6-His-tag, in a membrane association reaction followed by thermolysin treatment. After the reaction, proteins were separated on SDS–PAGE and used for Western analysis with antibodies raised against the 6-His-tag or POR. The intensity of the antigen–antibody label was similar before (Fig. 3C, lanes 2 and 6) and after (Fig. 3C, lanes 3 and 7) the

Fig. 3. Association reactions in lysates from pea chloroplasts using POR mutants. A and B: C-terminal deletion mutants of POR. The assays were performed with the in vitro translated transit peptide-free POR (Δ-TP) and with different C-terminal deletions. The samples were post-treated with (+) or without (−) thermolysin. C: Immunological detection using antibodies against the 6-His-tag (His) and POR (POR). Lanes 1–3 and 5–7 contain 6-His-tagged Synechocystis POR whereas lane 4 contains an EPIM fraction enriched in wheat POR. Lanes 2, 3, 6 and 7 show Synechocystis POR assembled to thylakoids in the presence of NADPH (2 mM) and MgATP (10 mM) and post-treated with (+) or without (−) thermolysin (0.2 mg/ml). In each lane, 10 μg of proteins is loaded. (i) Mature POR of about 40 kDa; (ii) membrane-associated POR protein of about 30 kDa; (iii) degradation product of POR of about 28 kDa following thermolysin treatment. D: Membrane association of the Cys-to-Ser POR mutants. The Ser substituted the Cys119, Cys281 and Cys308. The association reactions were performed in the presence of NADPH (2 mM) and MgATP (10 mM). The samples were post-treated with (+) or without (−) thermolysin (0.2 mg/ml). Δ-TP, in vitro translated transit peptide-free POR; TM, translation mixture; m, mature, processed form; dp, thermolysin degradation product of POR; 400, wild-type; 396, Δ396–400; 362, Δ362–400; 338, Δ338–400; 315, Δ315–400; 300, Δ300–400.
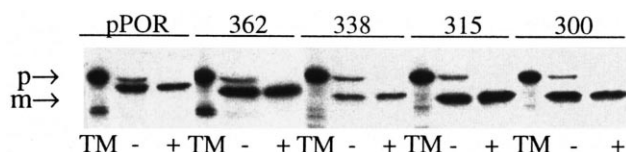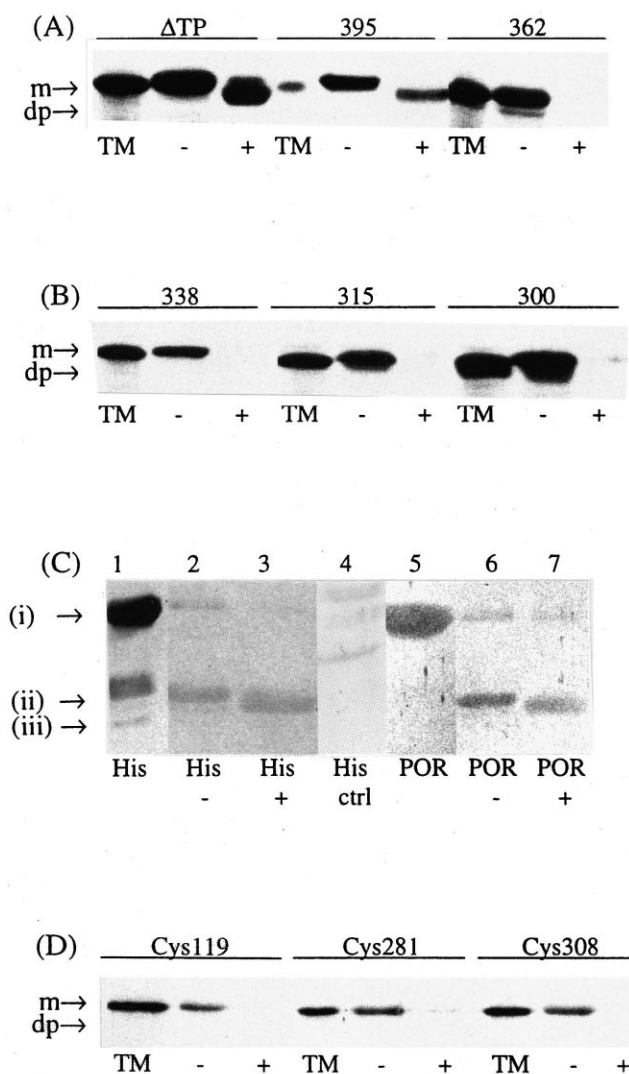

Fig. 2. Import reactions in pea chloroplasts using different C-terminal deletions of POR. The assays were conducted with the wild-type and the different C-terminal deletion pPOR proteins. The import reactions were performed in the presence of MgATP (10 mM) and after the assay the samples were treated with (+) or without (−) thermolysin (0.2 mg/ml). TM, translation mixture; p, precursor protein; m, mature, processed form; pPOR, wild-type pPOR; 362, Δ362–400; 338, Δ338–400; 315, Δ315–400; 300, Δ300–400.

thermolysin post-treatment, suggesting that proteolysis occurred at the C-terminus of POR. The 6-His-tag antibody did not react with endogenous wheat POR, which indicates the specificity against the 6-His-tag domain (Fig. 3C, lane 4).

In addition, the *Synechocystis* POR could be detected using the wheat POR antibody (Fig. 3C, lane 5) verifying that the 6-His-tag antibody did react to *Synechocystis* POR as the bands overlapped completely (Fig. 3C, lanes 2, 3, 6 and 7). We cannot exclude that the antigen–antibody label in Fig. 3C, lanes 6 and 7, also may correspond to endogenous POR.

3.2. Importance of Cys residues for the thylakoid association reaction of POR

The three Cys residues at the 119 (Cys119), 281 (Cys281) and 308 (Cys308) positions, relative to the first residue of the pPOR, were each substituted to Ser and the resulting three POR mutant proteins were used in membrane association studies (Fig. 1A). The rationale is as follows. Substitution of any of the Cys will to different degrees affect substrate (Pchlide) binding, cofactor (NADPH) binding and/or disulfide bridge formation. All of which may effect the membrane association reaction. As seen in Fig. 3D none of these single Cys substitution mutants were properly associated to the thylakoids, demonstrating that each and every one of the Cys is vital for POR to associate to the membrane.

4. Discussion

Nucleus-encoded chloroplast proteins have their organellar targeting information in the transit peptide that is necessary and sufficient for translocation into the plastid [27]. However, in addition to the transit peptide, the C-terminal part of the mature protein may also be engaged in the import reaction as shown for the small subunit of ribulose-1,5-bisphosphate carboxylase (pSS) through the PIRAC in the inner envelope membrane [16]. C-terminal deletions of pSS severely affected import and stimulated degradation of imported molecules in the stroma. This is in contrast to the C-terminal amino acid deletions of pPOR in the range 300–400, which did not affect the plastid translocation or post-import degradation.

Unlike the import into the chloroplast, the association with the thylakoid and assembly into complexes, such as for cytochrome and LHCP, exhibit a high degree of protein specificity. There are at least four different pathways into and across the thylakoid membrane. Lumenal proteins utilize the ΔpH pathway and the Sec pathway, whereas integral proteins utilize the signal recognition particle-like pathway and the spontaneous pathway [8,12]. For peripheral proteins associated on the stromal side of the thylakoid, such as POR, less information is available. Apparently, POR does not use any of the four suggested pathways even if similarities such as an ATP dependent membrane association can be found [4]. However, the final routing of POR to the thylakoids requires an intact C-terminal. Except for the PORΔ396–400 mutant, all C-terminal deletion mutants failed to resist thermolysin post-treatment, suggesting that amino acids in the range 362–395 are vital for membrane association. We conclude that the C-terminus, amino acids 300–400, of pea POR does not functionally interact with the translocation machinery across the envelope, whereas the amino acids 362–395 are important for proper association with the thylakoids. In addition, we propose that it is the C-terminus that is susceptible to protease post-treatment in vitro giving rise to the roughly 2 kDa degraded product seen in this paper and earlier studies [4,10,22]. This is interesting since although the C-terminus is required for thylakoid association, the extreme part of the C-terminus (2 kDa, about 19 amino acids) is not in a tight association to the membrane following final assembly.

It has been suggested that Trp residues are involved in the anchoring of POR [28]. The C-terminal part contains three Trp residues, Trp363, Trp365 and Trp390. Since Trp390 is within the thermolysin-sensitive part of POR, this indicates that Trp363 and Trp365 may be involved in the membrane anchoring of POR. It may be suggested that the C-terminal part containing these Trp residues form an anchor to the membranes. Studies of the accessibility of POR Trp for fluorescence probes indicate that the Trp residues are located at the polar heads of the lipids [29]. This is in line with the interpretation of the C-terminal as an anchor on the surface of the membrane.

Interestingly, POR mutants, containing substitutions of Cys to Ser, totally failed to associate to the thylakoids (Fig. 3D). The Cys119 is within the predicted nucleotide binding site of the enzyme, whereas the Cys281 is located within the predicted active site [22]. According to our earlier predictions [22], the Cys308 is not located within any of these two important sites. However, it is close enough to the predicted active site to exert an effect on Pchlide binding and/or reduction. Alternatively, the Cys308 may very well be required for maintaining the three dimensional structure of the protein.

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