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Irradiation-induced *in vivo* re-localization of NADPH-protochlorophyllide oxidoreductase from prolamellar body to stroma of barley etioplast

D. KOVACEVIC, D. DEWEZ, and R. POPOVIC*

Département de Chimie et de Biochimie, Centre TOXEN, Université du Québec à Montréal, CP 8888, Succursale Centre-Ville, Montréal (Québec), Canada H3C 3P8

Abstract

Distribution of NADPH-protochlorophyllide oxidoreductase (POR) in etioplast of etiolated barley leaf was studied by using Western blot analyses of etioplast fractions isolated on a sucrose gradient. When the leaf was exposed to light, POR content decreased in the etioplast inner membrane and prolamellar body sub-membrane fraction while it was simultaneously increased in the stroma. By using 77 K fluorescence spectroscopy analyzes, we found for irradiated etiolated leaf that the POR protein in the stroma was co-localized with chlorophyllide (Chlide) emitting at 678 nm. Re-localization of the POR-Chlide complex induced by irradiation suggests that POR participates in the pigment transport processes during early stages of the thylakoid membrane development.

Additional key words: chlorophyll(ide); chloroplast development; envelope; fluorescence spectra; *Hordeum vulgare*; prothylakoid.

Introduction

In leaves of dark-grown angiosperm, proplastids differentiate into etioplasts that accumulate photoactive ternary complex of photoenzyme NADPH-protochlorophyllide oxidoreductase (POR; EC 1.3.33.1), protochlorophyllide (Pchlide), and NADPH (Ryberg *et al.* 1993). POR enzyme catalyzes reduction of Pchlide to chlorophyllide (Chlide) as photon energy-requiring step in chlorophyll (Chl) biosynthesis (Griffiths 1978, Apel *et al.* 1980). In etiolated barley seedlings two distinct isoforms of POR enzyme, PORA and PORB, have been found. When etiolated leaves are irradiated, PORA content gradually decreases while PORB level persists (Holtorf *et al.* 1995). In dark-grown leaves POR enzyme appeared to be mainly associated with prolamellar body (PLB) sub-membrane structure of the etioplast inner membrane system (Ryberg and Sundqvist 1982a, Shaw *et al.* 1985). Immuno-electron microscopy studies of etiolated wheat leaves have suggested that POR enzyme translocates from PLB to PT sub-membrane when those leaves were irradiated (Ryberg and Dehesh 1986). Recently under *in vitro* conditions, when isolated barley etioplasts were exposed to short flash, PORA was released from the inner membrane into the stroma (Reinbothe *et al.* 2003b). However, POR re-localization process from PLB to stroma still remains to be investigated *in vivo*. We used Western blot analyzes of POR content in different etioplast fractions when dark-grown barley leaf was irradiated in order to provide evidence for irradiation-triggered POR translocation process in etioplast. Under those conditions, by using low-temperature fluorescence spectroscopy, the distribution of Pchl(ide) and Chl(ide) spectral forms in different etioplast compartments was also investigated.

Materials and methods

Plants: Barley (*Hordeum vulgare* L. cv. Chapais) seedlings were grown for six days on vermiculite moistured with tap water in the dark at 24 °C.

Isolation of total leaf proteins and etioplast inner membranes (EPIM): Etiolated leaf tissue was ground in liquid nitrogen and total leaf protein was extracted with the sample buffer of Laemmli (1970). EPIM isolation was based on the procedure described by Pursiheimo *et al.* (1998).

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*Corresponding author; fax: 514-987-4054, e-mail: popovic.radovan@uqam.ca
Abbreviations: Chl – chlorophyll; Chlide – chlorophyllide; EPIM – etioplast inner membranes; Pchlide – protochlorophyllide; PLB – prolamellar body; POR – NADPH:protochlorophyllide oxidoreductase; PT – prothylakoid.
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Isolation and fractionation of intact etioplasts: Etioplasts were isolated and fractionated by a modified method of Cline *et al.* (1981). The leaf tissue (150 g) was homogenized for 10 s in a blender with 1 500 cm^3 of ice-cold 25 mM HEPES-NaOH buffer (pH 7.5) containing 1 mM $MgCl_2$, 1 mM EDTA, 0.5 M sucrose, 5 mM aminocaproic acid, and 1 mM benzamidine. The slurry was filtered through two layers of *Miracloth* and centrifuged at 2 000×*g* for 10 min. The etioplast pellet was suspended in 8 cm^3 of homogenization buffer containing 25 % *Percoll* and then layered on the 40 % *Percoll* cushion. Intact etioplasts were further sedimented by centrifugation at 5 500×*g* for 25 min. Etioplasts were washed twice with the homogenization buffer, and then re-suspended in 0.12 cm^3 of the same buffer. The etioplasts were lysed with 1.4 cm^3 of 25 mM HEPES-NaOH buffer (pH 7.5) containing 1 mM $MgCl_2$, 1 mM EDTA, 5 mM aminocaproic acid, 1 mM benzamidine, and 0.3 mM NADPH for 15 min. Two ml of the lysate adjusted to 0.26 M sucrose was then layered on a sucrose step gradient made of 3 cm^3 of 1.20, 1.00, and 0.46 M sucrose in the lysis buffer. NADPH was added in all isolation media during the entire isolation procedure in order to prevent loss of the POR from the etioplast membranes (Ryberg and Sundqvist 1988). The gradient was centrifuged at 247 400×*g* in the *SW41* swinging-bucket rotor (*Beckman Coulter* ultracentrifuge) for one hour and then eleven fractions of 1 cm^3 were collected from top to bottom under the safe "green light". During the separation stroma remained at the top of the gradient in fractions 1 and 2. Low-temperature (77 K) fluorescence spectra revealed three pigment-enriched bands corresponding to fractions 2, 5, and 9. The fractions 5 and 9 are considered to be envelope-PT mixture and PLB containing, respectively, based on buoyant densities of PLB, PT, and envelope membrane (Cline *et al.* 1981, Ryberg and Sundqvist 1982a).

Fluorescence spectroscopy at 77 K: The spectra of 100 mm^3 aliquots of EPIMs and all the sucrose gradient fractions (1–11) were recorded between 600 and 750 nm with a spectrofluorometer *LS 50-B* (*Perkin Elmer*, Norwalk, USA) by using excitation wavelength of 440 nm (excitation and emission slits were 5 nm). The spectra were corrected for wavelength-dependent sensitivity of the fluorometer and normalized as indicated in figure legends.

SDS-PAGE and Western blot analyses: Proteins of the leaves, of the EPIMs, and of the sucrose gradient fractions (1–11) were separated by SDS-PAGE according to Laemmli (1970) and then electro-blotted onto a PVDF membrane (*Roche*). POR antibody-reactive proteins on the membrane were visualized by using polyclonal antibodies raised against barley POR (Barthélemy *et al.* 2000) and enhanced chemiluminescence (ECL) detection system (*Amersham*), and then recorded on X-ray film (*Fuji*).

Results and discussion

Changes of POR abundance and spectral properties (at 77 K) of EPIMs in irradiated etiolated barley leaves: When etiolated barley leaves were exposed to weak continuous irradiation of 10 μmol(photon) m^{-2} s^{-1} obtained by using a *Hansatech* tungsten halogen "white light" source and neutral density filters (*Corning*), a decrease of POR concentration in total leaf protein extract was evident after 120 min (Fig. 1*A*), while in total EPIM protein extract POR decrease was noticed already after 15 min (Fig. 1*B*). The decreasing trend of POR amount is due in part to proteolytic degradation of POR induced by irradiance (Kay and Griffiths 1983, Häuser *et al.* 1984). However, faster decrease of POR amount in the EPIM, compared to whole leaf, may be caused by the irradiation-induced release of a POR protein pool from the EPIMs. When etiolated barley leaves were exposed to a short (20 ms) saturating flash followed by darkness, the decrease of POR content in the EPIMs was noticed already after three minutes (Fig. 2*A*). However, when the leaves were maintained for 60 min in the dark after the flash, the POR content was again increased. Moreover, exposure of the leaves to a second saturating flash led again to fast decrease of POR content. On the other hand, no change of POR content was noticed in total leaf protein extract at 3 min following the flash (results not shown). The rapid decrease of POR content in the EPIMs may be interpreted to be caused by the irradiation-triggered POR re-distribution within the etioplast. This interpretation is consistent with results obtained for wheat EPIM and PLB etioplast membrane fractions showing partial release of POR protein upon irradiation (Ryberg and Sundqvist 1988).

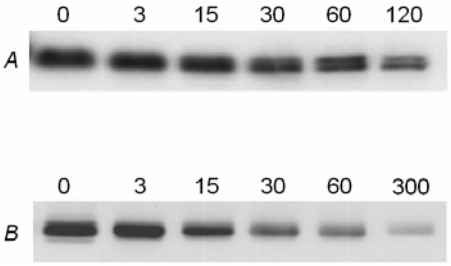

Fig. 1. Western blot analysis of POR protein in (*A*) total leaf protein (5 μg per lane) and (*B*) total EPIM protein (2.5 μg per lane) of etiolated leaves exposed to continuous irradiation (10 μmol m^{-2} s^{-1}). *The numbers* indicate time of irradiation of etiolated barley leaves [min].

The EPIMs isolated from non-irradiated etiolated barley leaves showed the presence of Pchlide with emission maxima at around 635 and 656 nm (Fig. 2*B*). Lower

656-nm photoactive Pchlide band intensity in the EPIMs compared to that in intact leaf is due to the absence of NADPH in the used isolation media according to Ryberg and Sundqvist (1982b). When etiolated barley leaf was exposed to the flash followed by 3 min of darkness, Pchlide emission of the EPIMs decreased and a 677-nm band appeared (Fig. 2*B*) indicating the formation of Chlide from Pchlide. After 60 min in the dark following the flash, Pchlide fluorescence emission was increased indicating the photoactive Pchlide regeneration process (Ryberg *et al.* 1993). At the same time, the shift of 677 nm fluorescence band to 680 nm resulted from formation of chlorophyll (Chl) forms with emission maxima at 685 and 695 nm which was shown by the difference spectrum analysis (Fig. 2*B*, *inset*). It has been suggested for Chl component emitting at 685 nm to originate from complex CP43 (Krause and Weis 1991) and for component emitting at 695 nm to be a vibrational band of $Pchlide_{633}$ (Böddi *et al.* 1992). The formation of Chl emission component at 685 nm coincides with the development of photosystem 2 (PS2) photochemistry seen 2 h following the flash in etiolated barley leaves (Franck *et al.* 1997).

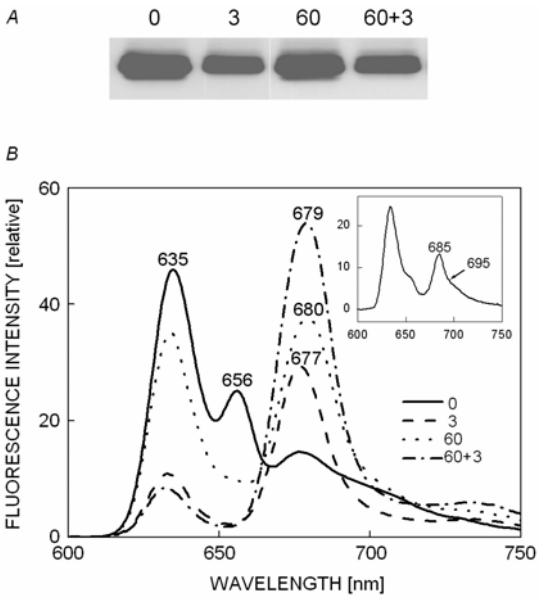

Fig. 2. (*A*) Western blot analysis of POR protein in total EPIM protein (2.5 µg per lane) of etiolated leaf which was 0: non-irradiated, 3: exposed to a short saturating flash followed by 3 min dark, 60: exposed to the flash followed by 60 min dark, 60+3: exposed to two flashes separated by 60 min of darkness and kept in the dark for 3 min after the second flash. (*B*) Low temperature (77 K) fluorescence spectra of aliquots of the same EPIM samples as in (*A*). Major Pchl(ide) and Chl(ide) bands are indicated by the wavelengths of their maxima at 635, 656, 677, 679, and 680 nm. The spectra are normalized on reflected stray radiation at 607 nm. *Inset*: difference spectrum between the spectra of EPIMs isolated from 60 min- and 3 min-dark-incubated flashed leaves. Newly assembled Chl-proteins are indicated by the maximum and the shoulder at 685 and 695 nm, respectively.

If second flash was applied after 60 min in the dark, Chlide formation occurred again supported by the regenerated photoactive Pchlide pool (Fig. 2*B*, spectrum 60+3).

POR distribution between etioplast fractions isolated from irradiated etiolated barley leaf: We investigated the irradiation-induced changes in distribution of POR and pigment forms among different etioplast compartments. For this purpose, intact etioplasts were isolated after different light/dark treatments of the leaves and three fractions (stroma, envelope-PT, and PLB) were analyzed.

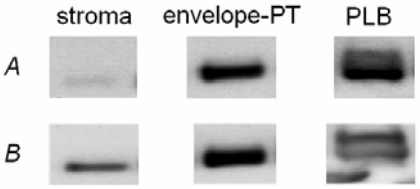

Fig. 3. Western blot analysis of POR protein in total protein (0.5 µg per lane) of sucrose gradient fractions. The gradient fractions (1–11, see Materials and methods) of (*A*) non-irradiated and (*B*) 20-min irradiated etiolated barley leaves were separated on identical gels (*Ready gel*, *Bio-Rad*) during a single run in the *Mini-protean II* unit (*Bio-Rad*) and the corresponding blots incubated with the same dilution of the primary anti-POR antiserum. Detection of the immuno-reactive bands was carried simultaneously to enable comparison of the POR band intensities between different blots. For clarity, only gradient fractions showing maximal fluorescence emission of either Pchlide or Chlide forms specific for stroma (fraction 2), envelope-PT (fraction 5), and PLB (fraction 9) etioplast compartments are presented.

The POR-related protein was recognized as a single band in stroma and envelope-PT etioplast fractions and as a doublet band in the PLB (Fig. 3). Double band may indicate PORA (lower band) and PORB (upper band) isoforms which exist in barley (Holtorf *et al.* 1995). Our results showed also a detectable presence of POR-related protein pool in the stroma fraction suggesting the presence of POR protein in the stroma *in vivo*. The presence of POR in the isolated stroma fraction might be caused also by contamination during isolation procedure. However, by using mild isolation protocol (hypo-osmotic lysis only) in the presence of NADPH we minimized this possibility (Ryberg and Sundqvist 1982b, Lindsten *et al.* 1988). When etiolated barley leaves were weak irradiated for 20 min, the change of POR protein distribution between different fractions of etiochloroplasts isolated from these leaves was found (Fig. 3*B*). POR protein of stroma appeared to be highly increased when etiolated barley seedlings were irradiated, while the irradiation did not induce any significant change of POR amount in the envelope-PT fraction. However, in the etiochloroplast fraction corresponding in density to PLB the lower band of the POR doublet was decreased relative to the upper band. We interpret these changes as indications that

photon energy induces the release of a POR fraction from the PLB into the stroma. Indeed, recently under *in vitro* conditions, PORA was released into stroma when isolated barley etioplasts were exposed to a short flash (Reinbothe *et al.* 2003b). To our knowledge, the change of the soluble POR content in the stroma under *in vivo* conditions has not yet been reported.

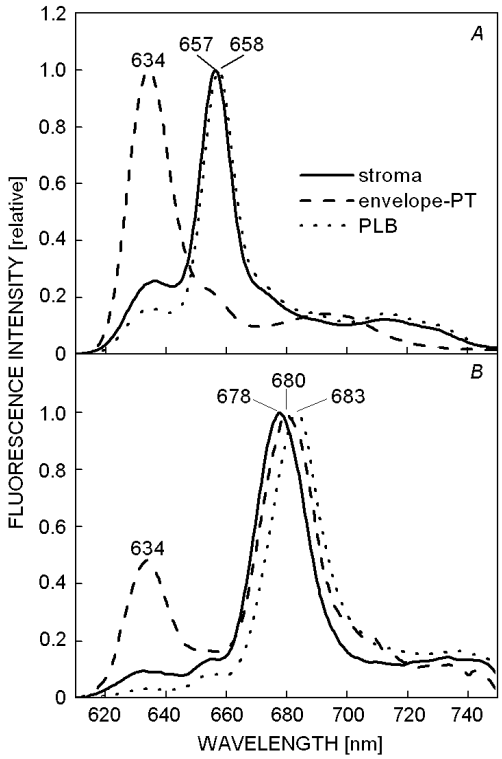

Fig. 4. Low temperature (77 K) fluorescence emission spectra of the etioplast fractions from (*A*) non-irradiated and (*B*) irradiated etiolated leaves. The spectra were normalized at their maxima.

Pigment distribution between etioplast fractions isolated from etiolated barley leaf: PLB contained mainly photoactive $Pchlide_{F657}$ (fluorescence emission maximum at 658 nm) while the envelope-PT fraction was characterized by the dominant presence of non-photoactive $Pchl(ide)_{F633}$ (Fig. 4*A*) in agreement with previous report (Ryberg and Sundqvist 1982b). We found photoactive $Pchlide_{F657}$ also in the stroma since it was co-localized with stromal POR on the sucrose gradient (Fig. 4*A*). Therefore, we suggest that stromal POR is in the form of aggregated ternary POR-Pchlide-NADPH complex (Böddi *et al.* 1989, Reinbothe *et al.* 2003a). Furthermore, the co-localization of the $Pchlide_{F657}$ and POR in the stroma indicates that presence of POR in the stroma is not caused by the contamination from the envelope membrane. This interpretation is supported also by evidence that POR was always co-localized with $Pchlide_{F633}$ form in envelope membrane (Joyard *et al.* 1990, Barthélemy *et al.* 2000). When etiolated barley leaves were weak irradiated for 20 min, Chl(ide) transformation process was indicated by the change of fluorescence emission spectra occurring in all etioplast fractions. However, this process was specific for every etioplast fraction since Chl(ide) forms with emission maxima at 678, 680, and 683 nm were characteristic for stroma, envelope-PT, and PLB, respectively (Fig. 4*B*). Co-localization of $Chl(ide)_{F678}$ and POR on the sucrose gradient may support the existence of the stroma-located POR-Chlide complex. Envelope-PT membrane spectral bands at 634 and 680 nm correspond to 633-636- and 679-680-nm bands found previously in the envelope membrane of mature spinach chloroplasts (Joyard *et al.* 1990) and PT sub-membranes of etiolated wheat seedlings (Lindsten *et al.* 1990). The 683-nm fluorescence band that we found for PLB fraction after 20 min of irradiation may be related to the major fluorescence band characteristic for etiolated leaves having the Shibata shift process completed (Ryberg *et al.* 1993).

In conclusion, the change in the distribution of POR enzyme in irradiated etiolated barley leaves indicated the existence *in vivo* of a rapid re-localization process of a POR pool from the PLB into the stroma compartment. We assume that the appearance of POR-Chlide complex in stroma of etioplast due to irradiation may reflect the POR-mediated transport process of the newly formed Chl(ide) toward the developing thylakoid membrane.

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