Distribution of the protochlorophyllide spectral forms in dark-grown pea seedlings, the effect of illumination and subsequent dark incubation on formation of pigment-protein complexes

PhD Theses

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Introduction

A key step of chlorophyll biosynthesis in higher plants is the protochlorophyllide (Pchlide) – chlorophyllide (Chlide) transformation. This reaction is catalized by the light dependent NADPH:protochlorophyllide oxidoreductase (LPOR; EC 1.3.1.33) enzyme. The enzyme reaction means in this case several parallel and simultaneous processes, because natively this enzyme forms macromolecular complexes with different structures and photochemical activities. The subunit of the enzyme is a ternary complex of the apoprotein, NADPH and Pchlide. These subunits can be in monomer, dimer or oligomer states arranged into the inner membranes of etioplasts. The complexity of the reaction is increased by the existence of isoforms of LPOR; in certain species only one, in others two, in *Arabidopsis* three isoforms have been described. These isoforms differ from each other in the regulation of their synthesis, activity and degradation.

The majority of results have been published about leaves of dark-grown seedlings, however, other organs than leaves contain chlorenchyma tissue – in case of etiolated plants prochlorenchyma. Spectroscopic and ultrastructural characterization of the epicotyl of dark-grown pea gave interesting new details to the description of etiolated organs and their greening process. Pea as experimental material is favourable because it contains only one the three above mentioned isoforms of the LPOR. Experimentally, the great variability of the ratios of Pchlide complexes seemed to be very interesting; the description of this variability and its characterization was missing in the literature.

A controversial question of the literature dealing with the greening process is, what happens to the LPOR enzyme after the reaction catalyzed by itself. According to certain authors, the LPOR macrodomains disaggregate after the reaction, then the ternary complex of the apoprotein, $NADP^+$ and Chlide falls apiece and the apoprotein will be decomposed. Other results show that the ternary complex regenerates and it can catalyze the Pchlide – Chlide reaction in several subsequent cycles. In this work, we have studied the effect of illumination and dark incubation in epicotyls of dark-grown pea.

Our research team often found a single band in the fluorescence emission region of chlorophylls in spectra of tissues differentiated at natural light conditions but under shading of other tissues. The conditions of the formation of this chlorophyll complex occurring even after a long period of dark storage were studied on leaves of pea seedlings.

Scientific background in the literature

The Pchlide - Chlide transformation is catalyzed in higher plants by the light dependent enzyme called LPOR (Griffiths 1975; 1978; Apel et al. 1980), which reduces the double bond between the 17th and 18th carbon atoms in the D ring of the porphyrine. (I studied only this enzyme in my work, therefore I use further on the simplified POR abbreviation when referring to this enzyme). Other photosynthesizing organisms – like gymnosperms (with the exception of ginkgo; Skribanek et al. 2008), pterophytes, mosses, algae and cyanobacteria – can synthesize chlorophyll also in the dark because they contain a light-independent enzyme marked as DPOR in the literature, in addition to POR. The DPOR enzyme is identical only in its function with POR, it is basically different in its genomic coding, molecular structure, subunit composition and catalytic mechanism (review: Masuda and Takamiya 2004). The majority of the POR enzyme is in prolamellar bodies (PLB) of etioplasts, but it is present also in prothylakoids (Dehesh and Ryberg 1985).

Despite the numerous data of the literature, lots of details have not been clarified yet: what conditions affect the stability of POR in the etiolated and then the illuminated tissues. This question is important because three isoforms of POR have been described – marked PORA, PORB and PORC, the synthesis and decomposition of which are under different regulations (review: Masuda and Takamiya 2004). The proteolytic decomposition of POR proceeding after illumination has been described (Kay and Griffiths 1983; Forreiter et al. 1990) and the sensitivity of complexes of POR with its substrates and products against proteases were studied (Reinbothe et al. 1995). According to their results, the pro-protein alone is rapidly decomposed when protein extraction of chloroplast was added. The PORA-Chlide complex is sensitive, proteases of plastids decompose it. On the other hand, the POR complexes with substrates, Pchlide and NADPH are resistant toward proteases. According to this theory, the newly formed product Chlide destabilises the POR-protein; its full proteolytic decomposition has been described. These authors supposed that the appearance of the POR specific protease is a light dependent process, what is connected to the formation of the chloroplast. Other works described regeneration processes of various types. According to one regeneration type, a single flash transforms the Pchlide sitting in the active site of POR into Chlide and in parallel, the NADPH is oxidized. The newly produced Chlide leaves the active site of the enzyme, which can thus bind a new Pchlide molecule favouring those bound to the POR oligomer but outside the active site. Simultaneously, the $NADP^+$ is reduced or replaced by new NADPH (Heyes et al. 2008). The POR remains active in several illumination cycles;

the photoactive complex can regenerate in many cycles (Franck et al. 1999). Other possibility is the quick regeneration of the 655 nm emitting complex via interconversions of other Pchlide forms within a few seconds. A dynamic equilibrium has been supposed among the Pchlide forms (Kahn and Nielsen 1974; Böddi and Franck 1997; Kósa et al. 2005), allowed by fluidity of the etioplast inner membranes. A slow regeneration process is known, too, in which monomer forms bound in the prothylakoid membranes are translocalized into the active sites of POR (Schoefs et al. 2000). The prerequisite of all these processes is the stability of the POR-protein; it must remain "intact" after the reaction.

In experiments where etiolated plant are illuminated the light sensitivity of these plants should be considered. Light sensitivity is remarkable in plants in which the non-photoactive (i.e. flash-inactive) Pchlide forms are dominating. In these plants, the Pchlide acts as photosensibilizer, it provokes the formation of singlet oxygen (Böddi et al. 2005; review: Reinbothe et al. 1996). The macrodomain arrangement of POR in PLBs of etiolated leaves inhibits photooxidation (Armstrong et al. 2000), because suitable amounts of NADPH are stored in the PLB membrane system. In organs of stem origin, thus also in epicotyls, only few and small sized PLBs occur (Böddi et al. 1994; McEwen et al. 1994; Skribanek et al. 2008), the NADPH content is low and the Pchlide is mainly in monomer state (Böddi et al. 1994; 1998; McEwen et al. 1994; Skribanek et al. 2000; 2008). In epicotyls of pea, light of very low intensity causes the partial degradation of Pchlide (Böddi et al. 2005). In the middle segments of pea epicotyls illuminated with strong light (500-600 μmol m^{-2} s^{-1}), singlet oxygen is produced what is followed by lipid peroxidation. This latter reaction results in turgor loss and irreversible wilting of the epicotyls (Erdei et al. 2005; Hideg et al. 2010). Production of superoxide and hydrogen peroxide was detected in the illuminated middle epicotyl sections. Even more, when hydrogen peroxide was injected into this region in the dark, a similar wilting was observed (Hideg et al. 2010).

In studies of etiolated plants the occurrence of chlorophylls were often found, besides the characteristic appearance of Pchlide. This can be explained that the embryo development has started still in light thus the cells of it contained chloroplasts. These chloroplasts could be transferred into dark formed cells via cell division in dark-germinated etiolated plants. This is proven by the gradual "dilution" of chlorophyll observed in the case of pea epicotyls: there is chlorophyll in the lowermost region of the epicotyls but it disappears from the upper regions (Böddi et al. 1999). Similar phenomenon can be observed in buds collected from the nature and forced in the dark (Solymosi et al. 2006), or similarly treated

twigs (Skribanek et al. 2000) and in fruits (Solymosi et al. 2007). The differentiation of the meristematic tissues starts in the light, and then they will be shaded by other tissues.

On the basis of these, we have chosen the following aims for this work: We wanted to study the biological variability of Pchlide forms occurring in pea seedlings by analysing the 77 K fluorescence emission spectra with statistical methods. Other aim was to study the stability and regeneration of the POR enzyme of pea epicotyls during and after bleaching with strong light. In the third part of our work, we studied the occurrence and the circumstances of the accumulation of the chlorophyll form with 675-682 nm fluorescence emission maximum. Plants were illuminated and then kept in the dark for long period.

Materials and methods

Dark germinated (etiolated) pea (*Pisum sativum* L. cv. "Zsuzsi") seedlings were used in this work. The distribution and biological variability of the Pchlide forms were studied in the lowermost, middle and uppermost segments of the epicotyls and in the apical leaves. 77 K fluorescence spectroscopic methods were used; the spectra were accumulated into a database which was analysed with statistical methods (average and the average of the absolute deviations of data points of their mean function) as well as with resolution of the spectra into Gaussian components. The stability of the POR under illumination and its regeneration processes in the dark were studied in the middle sections of pea epicotyls. 77 K fluorescence emission spectra were measured, the pigment contents were determined, polyacrylamide gel electrophoresis followed by Western blot and electron microscopic studies were done. The occurrence of the chlorophyll form occurring after long period dark storage was studied with 77 K fluorescence spectroscopy.

Results

The ratios of Pchlide complexes (spectral forms) were compared in different segments of epicotyls of pea seedlings of various ages. New statistical method was introduced to show the biological variability. The resolution of the average spectrum into Gaussian components confirmed the earlier results: Pchlide forms with 629, 636, 644 and 655 nm emission maximum are characteristic for the etiolated pea. Arranging the spectra of the leaves as well as of uppermost, middle and lowermost sections of epicotyls of plants with different ages into separate data groups, the average of the absolute deviations of data points of their

mean function was used. This special spectrum provided information about the biological variability of each Pchlide form what can give important reference basis when native tissue samples are studied spectroscopically. The 636 nm emitting form gave the greatest variability. The middle segment of epicotyls, which is the most light-sensitive, had the biggest variability.

The stability and regeneration ability of the POR was studied as follows: The pigments of pea epicotyls were bleached with strong white light, and then, after dark incubation, the regeneration of the system was examined. The spectral properties, pigment contents, the amounts of POR and the ultrastructure of plastids were studied during these processes. Despite nearly all pigments were bleached in the samples (at 0ºC the bleaching was faster) and the PLBs disintegrated in the etioplasts, nearly 50 % of the POR protein was detected. Surprisingly, the flash-photoactive 655 nm emitting oligomer complex regenerated first during the dark incubation. In parallel, the PLBs re-appeared. The regeneration of the shorter wavelength Pchlide forms was much slower. We concluded that a remarkable amount of the POR protein of pea epicotyls can remain stabile if the pigments and NADPH is removed from it. In addition, these protein subunits could stay in their original geometry needed for their oligomer arrangement thus the newly synthesised Pchlide molecules could enter into their active sites and could regenerate the 655 nm complex. Other possibility is that monomer POR subunits took up first the newly synthesized monomer Pchlide and NADPH but, under the force of PLB membrane lipids, they immediately aggregated.

A single and sharp emission band at 675-682 nm often appeared in fluorescence emission spectra of tissues of dark-stored plants or in tissues developed under shading of other, external tissues. We detected such band in different organs of a series of plant species, and we could form it experimentally: etiolated pea seedlings were shortly illuminated then kept in total darkness for 12 days. We think that this band belongs to special, stored chlorophyll-a complex.

Conclusions

1. The amounts of Pchlide forms and ratios among each other are organ and tissue specific. The leaves and the epicotyls of etiolated pea seedlings are different from this point of view. The ratio of forms is less variable in leaves than in the epicotyls of plants with different ages. The ratios of the Pchlide forms in the uppermost region of epicotyls resemble to those of leaves. The variability of Pchlide forms is the biggest in the middle section of the

epicotyls; especially the ratio changes of the 636 nm form are remarkable. The amounts of the two short wavelength form (with 629 and 636 nm emission maxima) showed strong correlation. Due to the low pigment content, the variability of the lowermost epicotyl region was very small, however, a band showing the presence of (probably embryonic origin) chlorophyll-a often appears. Considering these data, we suggest the study of spectra calculated from the average of the absolute deviations of data points of their mean function – in addition to the analysis of the mean spectra. These spectra show the biological variability of the emission bands.

2.) The middle section of etiolated pea epicotyl is a useful material for studying photo-oxidation stress reactions due to the dominance of monomer Pchlide forms: the Pchlide can be easily bleached in it upon illumination.

3.) The apoprotein of POR is not or is only partially affected in the photo-oxidation processes, even more the subunits of this enzyme maintain or rebuild their original supramolecular interactions. Therefore, the Pchlide molecules, *de novo* synthesized in the dark, build first into the oligomer POR complexes i.e. the flash photoactive 655 nm form regenerates; the regeneration of the short-wavelength forms is much slower process.

4.) The photodegradation causes reversible changes also in the inner membrane system of etioplasts: the regular membrane structure of PLBs disintegrates on the effect of light resulting in the bleaching of pigments, but it is re-formed during the dark-regeneration. The presence or absence of the 655 nm oligomer Pchlide form depends on the membrane structure of PLBs.

5.) In plant organs and tissues which differentiated under covering of other tissues, Pchlide forms appear under natural light conditions but often, besides them also chlorophyll is present. This chlorophyll is arranged into a spectral form having only a single emission band between 675-682 nm. This form can be experimentally prepared if etiolated seedlings are illuminated for a few hours and then kept in the dark for several days. This form must be a “stored” chlorophyll complex; understanding its structure and biological role requires further research work.

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