Theses of the PhD Thesis

STRUCTURES, *IN VIVO* AND *IN VITRO* INTERCONVERSION POSSIBILITIES AND WAVELENGTH-DEPENDENT PHOTOCHEMICAL REACTIONS OF PROTOCHLOROPHYLLIDE FORMS

Annamária Kósa

Supervisor: Prof. Béla Böddi
Head of Department

Eötvös Loránd University, Biology Phd School
School Leader: Prof. Anna Erdei
Experimental Plant Biology
Program Leader: Prof. Zoltán Szigeti

Eötvös Loránd University, Department of Plant Anatomy
Budapest
2010

Introduction

Chlorophylls (Chls) play basic role in the conversion of light energy into chemical energy in photoautotrophic organisms. They are present in pigment-protein complexes in living organisms; these complexes form photosystems in the chloroplasts. Connected to the photochemical reactions, a part of Chls is photooxidized or photodegraded irreversibly. Therefore the replacement of the degraded molecules in the Chl biosynthesis process is very important for the whole plant metabolism. A key step in Chl biosynthesis is the protochlorophyllide (Pchlide) - chlorophyllide (Chlide) transformation catalyzed by the NADPH:protochlorophyllide oxidoreductase enzyme (POR). This reduction reaction proceeds only on light in angiosperms. Thus dark-grown angiosperms accumulate Pchlide, and instead of chloroplasts, etioplasts differentiate. The inner membrane system is composed of prolamellar bodies (PLBs) with regular structure and of less regularly arranged prothylakoids (PTs). The main protein component of PLBs is the POR enzyme.

The POR enzyme is a ternary complex of the enzyme, NADPH and Pchlide. These units form various aggregates *in vivo,* which can be properly identified with spectroscopic methods. Plenty of results were published about the aggregates in the literatures. For example, it is well-known that the Pchlides bound in the active sites in the enzyme units are reduced into Chlide on ms time scale illumination. However, there are several open questions concerning these complexes, like the exact number of the naturally occurring Pchlide forms, their aggregation number and the factors influencing the aggregation of the complexes. There are only hypotheses about the structure and photoactivity of the forms containing monomer Pchlide.

In this work we studied the structures, photochemical activities and interconversion possibilities of Pchlide forms in etiolated stems and leaves *in vivo* and *in vitro*.

Materials and Methods

Plant materials

In the majority of the experiments we used pea (*Pisum sativum* L. cv. Zsuzsi) plants grown in hydroponic culture in the dark. 5-21 days old (5 - 35 cm long) seedlings were studied. Most in the experiments, the 2-3 cm long middle segment of the epicotyls were used. To study the photoactivity of Chlide complexes of etiolated leaves, 8 - 14 days old wheat (*Triticum aestivum* L. cv GK Öthalom) seedlings, 8-10 days old pea seedlings and isolated etioplast inner membranes (preparation method: Böddi et al. (1989)) were used.

To study the effects of cytokinin and nitrogen deficiency on the development of plastids of stems, shoot cultures started from pea embryos were applied. The plantlets were grown on Murashige-Skoog (Murashige and Skoog 1962), culture medium completed with

the following hormones: Benzylamino purine (1.0 or 0.3 mg/l), indolebutyric acid (0.1 mg/l) and naphtyl acetic acid (0.1 mg/l).

Homogenate preparation

Pea epicotyl (in some experiment leaf) pieces were homogenized in 0.05 M Na_2HPO_4 – KH_2PO_4 buffer (pH 7.2) containing 40% (v/v) glycerol and/or 40% (m/v) sucrose in the following conditions: 30 g (fresh weight) plant tissue, 1 cm^3 protease inhibitor cocktail (Sigma – Aldrich P9599) in 100 cm^3 buffer. The homogenates were stored in most experiments in a freezer at -14 °C for 4 days in the dark.

ROS detection with color-responsive indicators

The accumulation of hydrogen peroxide was detected with 3,3-diaminobenzidine (DAB) (Malmgren and Olson 1977). Superoxide was detected with nitroblue tetrazolium (NBT) staining (Beyer and Fridovich 1987).

Pigment extraction

The chlorophyllous pigments were usually extracted from epicotyls or leaves with 80% (v/v) acetone. In case of homogenates, diethyl ether was used for pigment extraction. The pigment concentrations and/or ratios were measured with spectrofluorometric methods. The calculations were done according to Brouers and Michel-Wolwertz (1983), using calibration curves.

Illumination

For continuous illumination with white light, tungsten lamp was used. Flash illumination was done with photo-flash apparatus Chinon-8000 (Yokohama, Japan) (energy output 160 J/ 0.002 s volt). For illumination with short-wavelength monochromatic red light He–Ne laser (S101 Globios MOM, Budapest, Hungary) was used emitting 632.8 nm light. The long-wavelength monochromatic red light-source was a laser diode (KWB Art. Nr. 0645-00, Stuhr, Germany) emitting 654 nm light. In all cases, the PFD values were set with neutral filters or diffusers, the PFD values were measured with LI-189-type Li-Cor photometer (Li-Cor Inc., Lincoln, NE).

Fluorescence spectroscopy

The fluorescence emission spectra were recorded with Jobin Yvon – Horiba Fluoromax 3 (Paris, Franciaország) type spectrofluorometer. When measuring 77 K spectra the samples were immersed into liquid nitrogen. The excitation light was usually 440 nm. The mean of 3-10 spectra were automatically recorded and calculated. The spectrofluorometric measurements were repeated on 2 – 20 samples collected from different plants.

Computer analysis of the spectra

The spectra were analysed with the software SPSERV V.3.11 (Bagyinka Csaba, MTA SZBK Institute of Biophysics). Calculations of difference spectra and deconvolution into Gaussian components were done. The 3D diagrams along with their topological visualization were done with the software SURFER Version 5.02 (Golden Software, Inc.).

Electron microscopy

The samples were fixed in glutaraldehyde (2%, 3 h) and osmium tetroxide (1%, 2 h), using 70 mM phosphate buffer (pH 7.2). In case of homogenates the samples were centrifuged (5 min, 16 000 g) after the fixation with glutaraldehyde. The pellet was mixed with cooling agar solution. The solid agar pieces were cut into pieces. These small pieces were put into osmium tetroxide solution. The next procedures were done according to Ryberg and Sundqvist (1982). The cuttings were studied with Hitachi 7100 transmission electron microscope (Hitachi Corp., Tokyo, Japan), the acceleration voltage was 75 KV.

Results and conclusions

The aim of this work was to study the spectral properties and the physiological roles of Pchlide forms and to get information about their molecular composition. Analysing the 77 K fluorescence spectra of dark-grown seedlings of wheat and pea, three short-wavelength forms were identified with emission maxima at 633 nm (P633) in wheat and at 629 (P629) and 636 nm (P636) in pea. In addition to these forms, 644 (P644) and 654-657 nm (P655) emitting long-wavelength forms were detected in both test-plants.

1. The variability of the Pchlide forms in the pea epicotyls

A returning problem of the experiments on pea epicotyls is the variability of the relative amounts of Pchlide forms and thus the 77 K emission spectra. In this case the calculation of average spectra hides the biological variability what can provide important information about the sample. To study this phenomenon the average of the absolute deviations of data points of their mean function (Microsoft Excel AVEDEV) was calculated for each data point of 100 fluorescence emission spectra. This calculation showed that the biological variation is the most remarkable in the middle region of epicotyls and the most variable was the emission band of P636. The variability of the Pchlide forms decreased with the age of the seedlings.

The results of this work show that the biological variation of plant samples must be considered and the calculation of the mean values alone may not be satisfactory.

2. Characterization of the short-wavelength forms

2a. The photochemical activity of the 636 nm emitting Pchlide form

The 77 K fluorescence excitation spectra of dark-grown pea epicotyls showed that the P636 has absorption (excitation) maximum at 632.5 nm. This gave an opportunity to study the

photoactivity of this form separately by using the 632.8 nm He-Ne laser light. A series of illumination of light periods between 100 ms and 10 min at room temperature and at -15 °C showed the direct phototransformation of this form into a 676 nm emitting Chlide form (C676).

The 632.8 nm emitting He-Ne laser light can transform a part of P636 directly; this shows that at least a part of this form is the ternary complex of POR-protein, NADPH and Pchlide, in which the Pchlide is bound in the active site of the enzyme.

2b. The photochemical activity of P633 in leaves and in PLB and PT preparations

The direct role of this form in Chl biosynthesis is a returning idea in works studying the greening process. Using the 632.8 nm emitting He-Ne laser, we excited the P633 in etiolated wheat leaves and in etioplast inner membranes. Flash-illumination of leaves with this light resulted in the accumulation of a C676 form, what could prove the direct photoreduction of P633. On the other hand, similar result was found at illumination with 654 nm monochromatic light of a laser diode. A combination of illumination with these lights of equal PFD (photon flux density) values was done and the pigments were extracted in acetone immediately. Calculating the relative ratio of Pchlide and Chlide, the rate of phototransformation was studied. The highest rate was found at 654 nm illumination; the 632.8 nm illumination caused much less Chlide production. No enhancement effect was found when the samples were illuminated with both lights.

These experiments gave no evidence for the direct phototransformation of P633.

2c. The structure and function of the 628-629 nm Pchlide form

No phototransformation (only bleaching) of the P628-629 form was observed in any of our experiments. The 628-629 nm band disappeared during several hours only when the etiolated tissues were illuminated with light of low PFD values. On the other hand, at high PFD illumination, this form provokes photooxidation reactions. In addition to the earlier described works indicating type II. photochemical reaction, we detected in this work type I. reaction, i.e. the production of hydrogen peroxide and superoxide.

This confirms the earlier data published in the literature: P629 is a non-POR-bound chromophore. It can serve as a pool from which the photo-transformed Pchlide forms can be regenerated. On the other hand, it is a sensitizer of type I. and type II. photochemical reactions.

3. Characterization of the 644 and 655 nm emitting Pchlide forms

3a. Structures of P644 and P655

We prepared pea epicotyl homogenate in glycerol and sucrose containing buffer. While the short-wavelength Pchlide bands were dominating in the fluorescence emission spectra of the epicotyls, incubation of the homogenate at -14 °C and/or freezing-thawing cycles (77 K – 293 K) in the dark, resulted in the accumulation of the 655 nm emission band and the decrease of the amplitude of the 636 nm band what proved the aggregation of the P636 into P655 took place in this experiment. In parallel, we observed the accumulation of P644. Flash illumination showed full photoactivity of P644 and P655.

The facts that the P644 and P655 forms could be accumulated or regenerated from P636 provided strong evidences of the "aggregation theory", i.e. that these forms are dimers and oligomers, respectively. At the same time, these experiments proved also that the P636 is a ternary complex of POR, i.e. a monomer unit of the longer wavelength forms.

3.b. Reconstitution after disaggregation

We could reconstitute the P655 from P636 in a different experiment, too. Epicotyl pieces were treated with freeze-thaw periods (77 K – 293 K) in the dark; this way we provoked the total disappearance of P644 and P655 forms. When homogenate was prepared from these epicotyl pieces, we observed the reconstitution of the 644 and 655 nm forms which were similarly flash-photoactive as in the intact epicotyls.

These results proved the aggregation nature of the P644 and P655.

3.c. Regeneration of the P655 in bleached samples (This part of the work was done in team work. The subject fits into my thesis indirectly. I took part in this work but the details will be used in another thesis-work.)

Strong white light bleaches almost all pigments in epicotyls of dark-germinated pea seedlings. When the bleached samples are incubated in total darkness at room temperature for about 4 hours, the P655 regenerates first, the regeneration of the shorter wavelength forms proceeds only after 18-20 hours dark-incubation. Interestingly, about the 50 % of the pigment-free POR protein is present in the bleached samples and after 4 hours dark-incubation its amount is about the same as in the control, not illuminated samples.

These results show that the POR-protein is stable in pea etioplast inner membranes. Even more, the native aggregated structure is preserved in the bleached samples, thus the newly synthesized PChlide molecules accumulate in the "empty" active sites of the oligomer POR complexes.

3.d. The phototransformation of P655 at very low light intensities

The reaction schemes summarizing the Pchlide - Chlide phototransformation usually indicate that the Pchlide oligomers transform into Chlide oligomers at flash illumination, and the latter

oligomers disaggregate during the Shibata-shift on a minute time scale. The laser illumination experiments in this work, however, showed the accumulation of 676 nm emitting Chlide or Chl-a form at flash illumination. In these experiments, 632.8 nm or 654 nm monochromatic lights of very low intensities were used. Analyses on the fluorescence spectra showed the transformation of P655 in wheat leaves, PLB, PT preparations and pea epicotyls.

On the basis of these results we hypothesised that light of very low intensities transform POR complexes only on the surface of the PLBs, the transformed complex "falls off" and immediately disaggregates into Chlide (or Chl-a) containing monomers. (We cannot close out the possibility of an immediate phytolization, i.e. the immediate Chl-a formation.)

4. Factors and conditions affecting the ratio of Pchlide monomers and oligomers *in vitro* and *in vivo*

4.a The effect of ATP on the formation of the 655 nm Pchlide form

ATP was reported to stimulate the formation of the 650 nm absorbing Pchlide form if added to homogenate of etiolated leaves. In addition, a hypothesis has been worked out stating that the POR activity is ATP-dependent what suggested special ATP bounding site(s) on the POR. To study these theories, we added ATP to pea epicotyls homogenates with a final concentration of 1.5 mM. We observed the relative increase of the fluorescence emission at 655 nm; this band disappeared on flash illumination.

ATP stimulates the aggregation of the ternary units of POR; this aggregation leads to the formation of the flash-photoactive P655.

4b. The effect of cytokinins on the formation of the 655 nm Pchlide form

Cytokinins were reported to stimulate the POR and Pchlide content as well as the formation of PLBs in etioplasts in certain plant species. To study the effect of exogenous cytokinins, organ cultures of pea were grown on culture medium containing various amounts of BAP. The embryos were dissected from the seeds thus the plants were dependent on the content of the culture medium. In these experiments, stems (nodal segments) were studied. We observed the increase of P655; at higher concentrations the fluorescence emission spectra of the stems were similar to those of leaves. In parallel, PLBs with regular membrane structure were found.

Cytokinins regulate the etioplast development in pea stem; external BAP stimulates the accumulation of P655 and in parallel, the formation of PLBs.

4c. The effect of nitrogen availability on the formation of the 655 nm Pchlide form

Nitrogen is a well-known substantial element for plants. In organ cultures grown as described above, we varied the nitrogen availability. If the culture medium did not contain available nitrogen source, PLBs did not occur in etioplasts.

In parallel with the increasing amount of nitrogen sources, the Pchlide content and the relative amount of P655 increased and the PLB membrane structures became regular.

References

Böddi B, Lindsten A, Ryberg M, Sundqvist C (1989) On the aggregational states of protochlorophyllide and its protein complexes in wheat etioplasts. Physiol Plant 76: 135-143

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 155: 473-497

Malmgren L, Olsson Y (1977) A sensitive histochemical method for light-and electron-microscopic demonstration of horseradish peroxidase. J Histochem Cytochem 25: 1280-1283

Beyer WF, Fridovich I (1987) Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal Biochem 161: 559-566

Brouers M and Michel-Wolwertz MR (1983) Estimation of protochlorophyll(ide) contents in plant extracts; re-evaluation of the molar absorption coefficient of protochlorophyll(ide). Photosynth Res 4: 265-270

Ryberg M and Sundqvist C (1982) Spectral forms of protochlorophyllide in prolamellar bodies and prothylakoids fractioned from wheat etioplasts. Physiol Plant 56: 133-138

List of publications containing the results of this thesis:

***A. Papers in refereed journals*:**

1., A. Kósa, Zs. Márton and B. Böddi (2005) Fast phototransformation of the 636 nm emitting protochlorophyllide form in epicotyls of dark-grown pea (Pisum sativum L.) Physiol Plant 124: 132-142

2., A. Kósa, Zs. Márton, K. Solymosi, K. Bóka and B. Böddi (2006) Aggregation of the 636 nm emitting monomeric protochlorophyllide form into flash-photoactive, oligomeric 644 and 655 nm emitting forms in vitro Biochim Biophys Acta Bioenergetics 1757: 811–820

3., A. Szenzenstein, A. Kósa and B. Böddi (2008) Biological variability in the ratios of protochlorophyllide forms in leaves and epicotyls of dark-grown pea (Pisum sativum L.) seedlings (A statistical method to resolve complex spectra) J Photochem Photobiol B: Biol 90: 88-94

4., A. Szenzenstein, A. Kósa, K. Solymosi, É. Sárvári and B. Böddi (2010) Preferential regeneration of the NADPH: protochlorophyllide oxidoreductase oligomer complexes in pea epicotyls after bleaching Physiol Plant 138: 102-112

5., É. Hideg, B Vitányi, A. Kósa, K. Solymosi, K. Bóka, S. Won, Y. Inoue, R.W. Ridge and B. Böddi (2010) Reactive oxygen species from type-I photosensitized reactions contribute to

the light-induced wilting of dark-grown pea (*Pisum sativum*) epicotyls Physiol Plant 138: 485-492

***B. Conference Proceedings*:**

1., A. Kósa and B. Böddi (2005) Dynamic interconversion and phototransformation processes of protochlorophyllide complexes during greening. Acta Biologica Szegediensis 49, 219-220 (Poster) 8th Hungarian Congress on Plant Physiology and the 6th Hungarian Conference on Photosynthesis. Szeged, Hungary.

2., Kósa A., Preininger É. and Böddi B. (2006) The effect of various extents of nitrogen deficiency on the etioplast differentiation in stems developed in pea (*Pisum sativum* L.) shoot cultures Proceedings pp. 84-88. oldal, ed.: Erzsébet Mihalik (Poster) XIIth *Symposium* of the *Hungarian Plant Anatomy*, Budapest

3., B. Böddi, K. Solymosi, A. Kósa, A. Szenzenstein, B. Vitányi and É. Hideg (2007) The occurrence of native monomer protochlorophyllide forms and their physiological roles Photosynthesis Research 91, 207

4., A. Kósa, Zs. Márton and B. Böddi (2003) Aggregation of NADPH:protochlorophyllide oxidoreductase (POR) subunits gives protection against photooxidation damage. Photosynthesis in a changing world (Photochange) Kolymbari, Crete, Greece, Poster: p 88. and oral presentation

5., Márton Zs., Kósa A. és Böddi B. (2003) Organisation of POR subunits into macrodomains cause increase in photochemical activity – P636 – P655 transformation *in vitro.* Vth. Hungarian Conference on Photosynthesis, Noszvaj, Poszter: p 6.

6., B. Böddi and A. Kósa (2004) Flash-phototransformation of the 636 nm emitting protochlorophyllide form in dark-grown pea (*Pisum sativum* L.) epicotyls. 13th International Congress of Photosynthesis, Montreal, Canada, Poster 643: p 219.

7., Kósa A. (2005) Role of monomer protochlorophyllide forms in greening. Native arrangement, aggregation and photochemical activity *in vivo* and *in vitro.* Oral presentation ont he general meeting of the Hungarian Society of Plant Physiology

8., Kósa A. and Böddi B. (2005) Structure, photoactivity and aggregation of monomer protochlorophyllide forms *in vivo* and *in vitro*. XXIIth. *Congress* of the *Hungarian Biophysical Society* Debrecen, (oral presentation), New pathway in protochlorophyllide photoreduction: flash-photoactivity of monomer NADPH:protochlorphyllide XXIIth. *Congress* of the *Hungarian Biophysical Society* Debrecen,, Poser p82.

9., A. Szenzenstein, A. Kósa and B. Böddi (2007) Statistical method to resolve fluorescence emission spectra measured from dark-grown pea seedlings Regional Biophysics Conference, Balatonfüred, Poster p. 116

10., A. Kósa and B. Böddi (2007) Phototransformation of protochlorophyllide forms under selective laser light illumination Regional Biophysics Conference, Balatonfüred, Poster p. 121

11., B. Böddi, K. Solymosi, A. Kósa, A. Szenzenstein and É. Hideg (2007) Native complexes of NADPH:protochlorophyllide oxidoreductase and their photo-activities 7th International Conference on tetrapyrrole Photoreceptors in Photosynthetic Organisms, Kyoto, Japan, 2007 Poster p. 25

12., K. Solymosi, K. Bóka, Z. Kristóf, B. Vitányi, A. Kósa, A. Szenzenstein, A. Skribanek, É. Hideg, B. Böddi (2009) Self-assembly of NADPH:protochlorophyllide oxidoreductase macrodomains in vivo and in vitro European Biophysics Congress (EBSA), Genoa, Poster

13., B. Vitányi, A. Kósa, K. Solymosi, B. Böddi (2010) What's hidden under the surface? Etioplast development under natural condition. Magyar Mikroszkópos Konferencia, Siófok, Előadás (B. Vitányi)

14., B. Böddi, K. Solymosi, A. Kósa, B. Vitányi, É. Hideg (2010) Chlorophylls and their precursors in food plants. Proceedings of the 6th International Congress on Pigments in Food; Chemical, Biological and Technological Aspects, Budapest, Hungary, ISBN 978-963-9970-04-5 Plenary lecture (Béla Böddi), p. 42