Institute of Plant Biology
Biological Research Center

Faculty of Science
József Attila University

Ph.D. Thesis

ON THE MECHANISM OF PHOTOSYSTEM II REACTION CENTRE D1 PROTEIN DEGRADATION INDUCED BY EXCESS VISIBLE- AND ULTRAVIOLET-B LIGHT IN ISOLATED SYSTEMS FROM GREEN PLANTS

by

CORNELIA SPETEA

Ph.D. Supervisor: Dr. IMRE VASS
Institute of Plant Biology
Biological Research Center

Szeged BRC

Szeged 1996

Other publications not used in the thesis:

Hideg, É., **Spetea, C.** and Vass, I. (1994) Active oxygen and free radical production in spinach thylakoids during photoinhibition. Evidence from EPR spectroscopy. In: *Frontiers of Reactive Oxygen Species in Biology and Medicine* (Asada, K. and Yoshikawa, T. Eds.), pp. 33-34, Kluwer Academic, Dordrecht, The Netherlands.

Hideg, É., **Spetea, C.** and Vass, I. (1995) EPR spectroscopy detection of active oxygen and free radicals in thylakoids exposed to photoinhibition. *Acta Phytopathol. Entomol. Hung. 30*, 51-57.

Vass, I., **Spetea, C.**, Hideg, É., Barbato, R. and Petrouleas, V. (1995) Ultraviolet-B radiation induced damage to the function and structure of photosystem II. *Acta Phytopathol. Entomol. Hung. 30*, 47-49.

Hideg, É., **Spetea, C.** and Vass, I. (1995) Are superoxide radicals involved in acceptor side induced photoinhibition? In: *Photosynthesis: from Light to Biosphere* (Mathis, P. Ed.) *Vol. IV*, pp. 287-290, Kluwer Academic, Dordrecht, The Netherlands.

Vass, I., **Spetea, C.**, Sass, L., Hideg, É. and Petrouleas V. (1995) Ultraviolet-B radiation induced damage to the function and structure of photosystem II. In: *Photosynthesis: from Light to Biosphere* (Mathis, P. Ed.) *Vol. IV*, pp. 461-464, Kluwer Academic, Dordrecht, The Netherlands.

Vass, I., Sanakis, Y., **Spetea, C.** and Petrouleas, V. (1995) Effects of photoinhibition on the Q_A-Fe^{2+}complex of Photosystem II studied by EPR and Mössbauer spectroscopy. *Biochemistry 34*, 4434-4440.

Spetea, C., Hideg, É. and Vass, I. (1996) The quinone electron acceptors are not the main sensitizers of UV-B induced protein damage in isolated photosystem II reaction centre- and core complexes. *Plant Sci., in press.*

Vass, I., Sass, L., **Spetea, C.**, Bakou, A., Ghanotakis, D.F. and Petrouleas, V. (1996) UV-B induced inhibition of photosystem II electron transport studied by EPR and chlorophyll fluorescence. Impairment of donor and acceptor side components. Submitted to *Biochemistry*.

INTRODUCTION

Photosynthesis is the conversion of solar energy into chemical energy, followed by CO_2-fixation. In green plants, photosynthesis takes place in the chloroplast. The light is absorbed by two photosystems (I and II), embedded in the chloroplast thylakoid membranes. When much more light is absorbed than can be used for CO_2-fixation, inactivation of photosynthesis occurs. Thus, sunlight can be also harmful to plants, the magnitude of the effect depending on both quality and quantity of light. An excess of photosynthetically active visible radiation (PAR, 440-700 nm) or even a low dose of ultraviolet-B (UV-B, 280-320 nm) light can adversely affect plant growth as well as photosynthetic capacity, with important ecological and agricultural consequences.

The complex damage to the photosynthetic apparatus brought about by exposure to excess visible light is generally known as **photoinhibition** and is a common feature of all oxygen-evolving photosynthetic organisms (green plants and cyanobacteria). Although there are several other symptoms, the primary target of light-induced damage has been shown to be the **water-splitting Photosystem II** (PS II) complex (*Fig. 1*). Illumination of PS II by excess light results in the inactivation of its electron transport followed by the selective degradation of reaction centre II (RC II) **D1 protein,** which is ligating most of the active PS II redox components.

The primary event in photoinhibition is the destruction/release of redox components located at the acceptor- or donor side of PS II electron transport system. Excess visible light is required to damage the RC II chlorophyll (P_{680}) which directly and/or through active oxygen formation triggers the D1 protein for degradation. The actual cleavage is light-independent, leading to fragmentation patterns specific for the acceptor- and donor side type of PS II photoinactivation (*Fig. 2*). The importance of these well-characterized photoinhibitory mechanisms in isolated systems is not fully clarified in the process of photoinhibition *in vivo*. The stroma-exposed cleavage site of the D1 protein, characteristic to the acceptor side conditions, was shown to be preferentially activated in most of the reports,

though recently, indications for a predominant occurrence of the donor side type of photoinactivation have been also observed. Destruction of the central D1 polypeptide leads to a partial disassembled state of PS II. Under *in vivo* conditions, the repair process of the damaged PS II is functional, consisting of: synthesis and re-insertion of a new D1 protein, re-ligation and re-activation of the PS II redox components. When D1 synthesis and replacement can not compensate for its damage and degradation, photoinhibition occurs.

UV-B radiation is known to be even more harmful to oxygenic photosynthesis than visible light. The stratospheric ozone depletion over Antarctica but also Australia and Northern Europe, has led to an increasing interest directed to understanding the molecular targets and mechanisms underlying UV-B effects on PS II. The most susceptible parts of PS II have been reported to be UV-B absorbing redox components involved in the electron transfer on the donor side, like the Tyr_Z, Tyr_D and the water-oxidizing Mn cluster, or the quinone acceptors (Q_A, Q_B and plastoquinone pool) located on the opposite surface of the thylakoid membrane. An other assumption was that UV-B radiation could attack the PS II reaction centre itself. So, there are contradictions in estimating the relative contribution of redox cofactors to the overall loss of PS II activity caused by UV-B light. The RC II D1 and, to a lesser extent, D2 proteins are specifically degraded; therefore, the UV-B effect can not be assigned to an overall PS II protein damage. Depending on the experimental conditions, both donor- and acceptor side components have been suggested to be involved in the damage to the D1 protein. Degradation pathways specific to visible-light-induced photoinhibition are very well characterized. The pattern of cleavage induced by UV-B light is much less studied and it is not known if it proceeds according to the same or different pathway as found for the visible light. It is also not clear if the degradation occurs via proteolytic activity or by direct photocleavage through indirect effects, such as free radical formation which also increases at enhanced UV-B levels.

Understanding the molecular processes giving rise to the vulnerability of PS II to light and also the subsequent repair/protection system is a central area of present-day photosynthesis research.

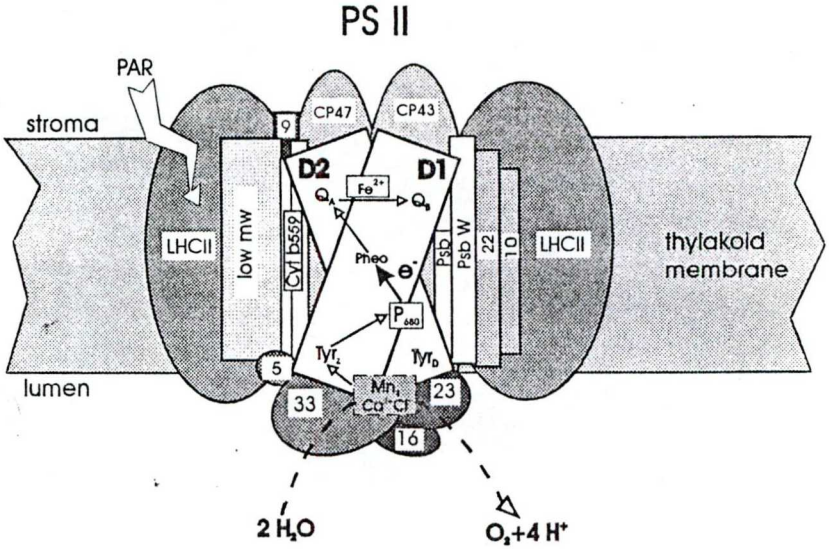

Figure 1.

STRUCTURE AND FUNCTION OF THE PHOTOSYSTEM II COMPLEX.

Protein subunits are indicated with their names or molecular weights (kDa). The central part of the figure shows the D1/D2 heterodimer with the bound electron carriers: the primary chlorophyll donor (P_{680}), the primary pheophytin acceptor (Pheo), the first and second quinone acceptors (Q_A and Q_B), the non-haem iron (Fe^{2+}), the tyrosine donor to P_{680} (Tyrz), the accessory tyrosine donor (Tyr_D), the catalytic Mn_4 cluster of water oxidation and its cofactors (Ca^{2+}, Cl^-). The reaction centre II also contains the α- and β-subunits of cyt b_{559} and the products of psbI and psbW genes. Other protein components with various roles in the function of PS II are the following: the light-harvesting complex (LHC II), the chlorophyll-binding proteins CP47 and CP43, the extrinsic proteins of the water-splitting system (33, 23 and 16 kDa), the 22 and 10 kDa intrinsic proteins, the 9 kDa phospho-protein, the extrinsic 5 kDa polypeptide and some low molecular weight proteins (low mw). The filled arrow represents the primary charge separation, whilst the empty arrows symbolize the secondary electron transfer reactions that participate in the stabilization of the charges. The broken arrow indicate the essential event of water decomposition occurring on the lumenal side of PS II.

OBJECTIVES

The molecular mechanisms of D1 protein degradation induced by excess visible- and UV-B light are the main topics of this thesis. The following questions are addressed:

- *What is the correlation between D1 protein degradation and the formation of active oxygen species under visible-light-induced photoinhibition?*
- *What is the role of low pH in visible-light-induced photoinhibition and D1 protein degradation?*
- *What are the characteristics of UV-B induced D1 protein cleavage?*
- *What are the roles of quinone electron acceptors and PS II donor side components in the UV-B induced D1 protein degradation?*
- *Is the pattern of D1 protein cleavage specific only to the B-domain of UV spectrum?*

EXPERIMENTAL MATERIAL AND PROCEDURE

It is assumed that loss of the photosynthetic capacity in plants can occur at any light intensity in the field, but under low light destruction is masked by the repair process. However, under higher excess light conditions, photoinhibiton becomes apparent because the rate of D1 protein degradation exceeds the rate of re-synthesis leading to a rapid disappearance of all PS II subunits. In isolated systems, the equipment for repair is missing/not functional and, thus, the D1 loss and fragmentation can be easily detected. Therefore, isolated systems provide a useful experimental tool for studies on the mechanism of D1 protein degradation. Our experiments have been carried out in isolated systems from spinach leaves:

- **thylakoid membranes**, containing PS I, PS II, cytochrome (cyt) b/f and CF_0/CF_1 complexes;
- **PS II-enriched membranes** (*Fig. 1*);
- **PS II core complexes**, which are PS II complexes depleted of LHC II and the extrinsic proteins of 23 and 16 kDa (*Fig. 1*). This is the minimum PS II preparation that can evolve oxygen and release protons;
- **RC II complexes**, containing the D1/D2 heterodimer, the α- and β-subunits of cyt b_{559} as well as the *psb*I and *psb*W gene products (white areas from *Fig. 1*). This preparation can perform only the primary photochemistry because the secondary electron donors and acceptors are not retained.

The **irradiation** experiments have been performed with high PAR or UV-A (365 nm), B (312 nm) and C (254 nm) light for various periods of time at 22°C or 4°C. Untreated and Tris-washed (inactive in oxygen evolution) thylakoids were used as model systems for acceptor- and donor-side-induced photoinactivation, respectively. For acceptor side conditions in less intact systems, like PS II core complexes, 5 mM $CaCl_2$ was added to a 50 mM Mes buffer (pH 6.0) in order to stabilize the donor side of the particles. Where indicated, donor side conditions were obtained by replacement of the Mes buffer with 50 mM Tris (pH 8.0), in order to perturb the donor side, and by the addition of 0.2 mM DBMIB to ensure efficient electron transport at the acceptor side. In experiments regarding the role of quinones in the UV-B related damage, the selective extraction of the Q_B quinone electron acceptor has been performed in isolated thylakoids.

Light-induced changes in the PS II protein structure have been followed using **SDS-polyacrylamide gel electrophoresis** and **immunoblotting** techniques. Two different types of polyclonal antibodies: anti-D1 (provided by Dr. R. Barbato, Padova, Italy) and anti-D1C (provided by Dr. P. Nixon, London, U.K.) have been employed to immunodetect the loss and the carboxyl (C)-terminal breakdown products of the D1 protein, respectively. Where indicated, steady-state **oxygen evolution** in the control and illuminated samples has been measured in order to compare the time courses for light-induced damage to the PS II function and structure.

RESULTS AND DISCUSSION

Visible light stress of photosystem II

The possible role of active oxygen species (singlet oxygen, hydroxyl radical and superoxide radical) in the visible-light-induced triggering and/or cleavage of the D1 protein has been studied in isolated thylakoids. Exposure of photosynthetically active thylakoids to photoinhibition brought about impairment of PS II acceptor side accompanied by production of singlet oxygen ($^1O_2$) as detected by EPR spectroscopy. In contrast to this, when Tris-washed thylakoids were irradiated with high PAR, hydroxyl radicals dominated as active oxygen species and no $^1O_2$ was formed. Comparing the kinetics of D1 protein

damage with that of singlet oxygen or free radical EPR-trapping demonstrated that during the progress of both types of photoinhibition, the production rate of the dominant active oxygen form increases prior to the D1 protein loss (**I, II**). Therefore, the radicals formed in the damaged PS II centres may trigger the D1 protein for degradation.

Superoxide radicals are produced in illuminated thylakoid membranes even without stress conditions. Therefore, we investigated whether they also contribute to the observed D1 protein degradation during acceptor-side-induced photoinhibition of isolated thylakoids and PS II core particles. Artificially generated superoxide radicals (from the photoreaction of riboflavin) did not cause a dramatic increase in the low- or high-light-induced inactivation of oxygen evolution and D1 protein loss in thylakoids as compared to the experiments performed in the absence of riboflavin (**III**). This indicates that the superoxide radicals, produced most likely within PS I, do not participate in the observed acceptor side type of light-induced D1 degradation. However, the externally added superoxide may trigger a new pathway of D1 cleavage. This possibility has been investigated by studying the D1 breakdown pattern in PS II core complexes. Surprisingly, the well-known pattern composed of 10 and 16 kDa C-terminal fragments of the D1 protein (*Fig. 2*) was not enhanced by externally-generated superoxide. Instead, a new larger doublet of 17-20 kDa products was detected suggesting some artificial cleavage sites in the lumenal loop between the C and CD helices and in the transmembrane B helix (**III**). Therefore, our results imply that even though the presence of exogenous superoxide accelerates D1 protein degradation, this is due to an additional process and not the enhancement of acceptor-side-induced reactions.

In photoinhibited leaves, the proton gradient created across the thylakoid membrane exceeds that required for ATP synthesis; thus, the thylakoid lumen becomes more acidic. In a significant number of reports, on the *in vivo* light-induced acidification of the thylakoid lumen are based several protective mechanisms by which excess excitation energy could be dissipated within the light-harvesting complex or RC II. However, low pH is among the conditions which inhibit water oxidation. Therefore, there are also some suggestions that

light-induced acidification of the thylakoid lumen might induce a very efficient donor side photoinactivation. Photoinhibitory experiments have been performed at various pH values in isolated photosynthetic systems, in an attempt to understand the effect of the low pH on the mechanism of PS II photoinactivation and of D1 protein degradation. Our results suggest that lowering the pH leads to the inhibition of oxygen evolution followed by cleavage of the D1 protein by gradually converting the mechanism of photoinhibition from the acceptor side type, at pH 7.0, to the donor side type, at pH<4.5 (**IV**). The preferential accumulation of 16 and 23-24 kDa D1 C-fragments during photoinhibition of PS II core complexes at pH 4.5, indicates the cleavage of the protein on the loops connecting the C-CD and A-B helices, respectively (*Fig. 2*). The light-activated D1 cleavage sites at low pH are exposed on the lumenal side of the thylakoid membrane, where actually the *in vivo* acidification occurs. Therefore, we also suggest that the down-regulation models based on the pH gradient could not provide protection against PS II photodamage; instead, low pH could rather induce a very efficient donor side photoinhibition.

Ultraviolet-B light stress of photosystem II

UV-B radiation adversely affects the functional and structural integrity of the photosynthetic apparatus. However, the mechanism of damage, especially to PS II complex, is far from being elucidated. UV-B induced fragments of the D1 protein have been observed previously and assigned to a cleavage in the stromal loop connecting the D and DE helices of the protein (*Fig. 2*). Therefore, a D1 degradation pathway similar to the one of acceptor-side-induced photoinhibition was proposed to occur. In the present study, some previously undescribed breakdown fragments generated by the action of UV-B radiation were identified as evidence for a different degradation mechanism than under visible-light-photoinhibition. When isolated spinach thylakoids were exposed to UV-B light, the D1 protein was gradually lost, but a 20 kDa C-terminal fragment was accumulated (**V**). In addition to this product, in oxygen-evolving PS II core complexes a larger UV-B induced D1 C-fragment of 25 kDa was also immunodetected. The breakdown pattern suggests cleavage sites at the middle or lumenal end of the second transmembrane helix (*Fig. 2*), which have not been observed under visible-light-induced photoinhibition. However, the proposed

UV-B mediated cleavage sites are located closer to those characteristic to donor-side-induced photoinhibition than to the ones activated under acceptor side photoinhibitory conditions. Production of the fragments during UV-B irradiation even at low temperature or in anaerobiosis indicates a direct D1 photocleavage instead of a proteolytic degradation (V).

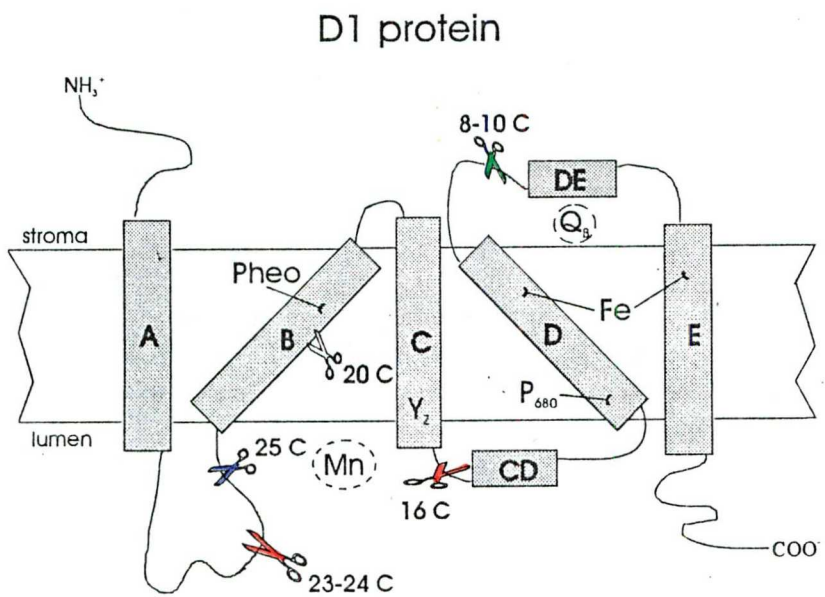

Figure 2.

VISIBLE- AND ULTRAVIOLET LIGHT-ACTIVATED CLEAVAGE SITES OF THE D1 PROTEIN. *According to the folding model predicted by Svensson et al. (1990), the D1 protein has five transmembrane (A-E) and two membrane-parallel (CD and DE) α-helices on which the indicated PS II redox components are bound (P_{680}, Pheo, Mn, Q_B and Fe) or contained (Tyrz). The scissors show the possible cleavage sites activated under acceptor-side-induced photoinhibition (green), donor-side-induced photoinhibition (red) and UV irradiation (blue). The corresponding C-terminal fragments of the D1 protein are also indicated to illustrate the difference in the cleavage mechanism triggered by visible- and UV light.*

The role of UV-B absorbing PS II redox components bound on the D1 protein (see 'Introduction') in sensitizing the observed cleavage sites, was also investigated in this work. Although it is possible that the quinone acceptors, Q_A and Q_B, are involved in UV-B absorption, the D1 protein is cleaved at sites located quite far from the Q_B-binding niche (*Fig. 2*). This observation is supported by the fact that the same 20 and 25 kDa C-fragments were detected in intact and Q_B-depleted thylakoids (**VI**). The UV-B induced loss of the D1 protein was similar in the presence and in the absence of Q_B. Therefore, contrary to earlier assumptions, the cleavage of the D1 protein does not require the presence of Q_B as UV-B sensitizer.

The effect of quinones in the UV-B induced protein damage was further investigated in isolated RC II complexes. Even though these preparations do not retain any bound quinones, both the D1 and D2 proteins, but not the cyt b_{559}, were damaged (**VII**). The time course of the protein loss was about the same in the absence and in the presence of artificial acceptors like DBMIB (**VII**). However, UV-B irradiation of RC II complexes resulted in the appearance of several fragments of the D1 protein in the 14-25 kDa range only in the presence of DBMIB (**VIII**). Low temperature or absence of oxygen did not prevent the degradation. Despite the quinone mediated UV-B damage of the D1 protein in reaction centre II complexes, there is increasing evidence that in more intact systems the PS II donor side is involved in the degradation process (**VI**).

The 20 kDa D1 fragment could not be detected upon UV-B irradiation of thylakoids lacking the water-splitting manganese cluster, in agreement with the recent results of Barbato *et al.* (1995). However, this fragment was restored not only by addition of $MnCl_2$ but also by other artificial electron donors to PS II, such as diphenylcarbazide (in preparation). We suggest that a functional PS II donor side electron transport is required for sensitizing the observed D1 cleavage.

UV-B mediated cleavage of the D1 protein is also likely to involve highly oxidizing species. Since the donor side of PS II is an important target of UV-B and the active oxygen production is similar in UV-B irradiated and donor-side-

photoinhibited PS II, we propose a model in which UV-B impairs the donor side electron transport of PS II, then provokes donor-side-induced photoinhibition and hydroxyl radical production. The fragmentation of the D1 protein in donor-side-induced photoinhibition (23-24 kDa C-terminal products) and in UV-B damage (20 and 25 kDa C-terminal products) were found to be very similar but not identical (in preparation). Thus, donor-side-induced photoinhibition can not fully explain the observed D1 fragmentation pattern mediated by UV-B light.

An alternative source of hydroxyl radicals could be the UV-B mediated decomposition of hydrogen peroxide, which can be an intermediate product of water oxidation. A possible direct involvement of free radicals in the fragmentation of the D1 protein was supported by the fact that artificially generated hydroxyl radicals, by metal-catalyzed reduction of hydrogen peroxide, or of superoxide radicals, from illumination of riboflavin, could activate the same cleavage sites of the D1 protein as UV-B irradiation (in preparation). Indeed, hydroxyl radicals have been EPR-detected in UV-B exposed thylakoids, and superoxide could be an intermediate step in their induction. Therefore, we suggest that UV-B induced free radicals may also participate in the cleavage process or could be only by-products of the observed UV-B damage.

The loss and fragmentation pattern of the D1 protein showed the same dose-dependence at two different UV-B intensities (50 and 7 $\mu E \cdot m^{-2} \cdot s^{-1}$) suggesting that our results are relevant to, and can be extrapolated to lower UV-B intensities occurring under natural conditions (**VI**). Especially, the detection of the same 20 kDa D1 product following irradiation with low UV-B is very important to emphasize that the UV-B induced damage is not related to some 'overreduction'/'overoxidation' process and it proceeds according to a unique mechanism regardless of light intensity.

The fragmentation pattern of the D1 protein detected under UV-B irradiation is not specific to this UV domain exclusively since it was found to be also induced by UV-C and even UV-A light although to different extents (in preparation). These results suggest that there is no specific UV-B sensitizer for the observed D1 cleavage. The mechanism of damage is most likely uniform for the entire UV spectrum. The only difference could be in the respect of light

energy (the highest for UV-C and the lowest for UV-A) and consequently, in the efficiency of the damage process.

CONCLUSIONS

The most important findings in this thesis could be summarized as follows:

- *Triggering and/or cleavage of the D1 protein during acceptor- and donor-side types of* ***photoinhibition*** *is related to* $^1O_2$ *and* $HO^{\cdot}$ *oxidative damage, respectively (***I, II***).*
- *Superoxide radicals are not the main promoters of acceptor side mechanism of visible-light-induced D1 protein degradation (***III***).*
- *Lowering the pH during photoinhibition in isolated systems accelerates photoinhibition of oxygen evolution followed by D1 protein degradation by gradually converting the mechanism of photoinhibition from the acceptor side type, at pH 7.0, to the donor side type, at pH<4.5 (***IV***).*
- ***UV-B*** *mediated cleavage sites at the middle and lumenal end of the second transmembrane helix of the D1 protein are different from those induced by visible light photoinhibition (***V***).*
- *UV-B induced cleavage of the D1 protein does not require* Q_B *as UV-B sensitizer in oxygen-evolving PS II preparations (***VI***).*
- *The D1 and D2 proteins are degraded in quinone-free RC II complexes upon UV-B irradiation (***VII***), but the pattern of D1 fragmentation is different from that observed in more intact systems (***VIII***).*
- *The mechanism of UV-B damage implies D1 protein photocleavage instead of a proteolytic process (***V, VII, VIII***).*
- *Cleavage of the D1 protein is triggered by UV-B mediated inactivation of the PS II donor side and/or free radical production in oxygen-evolving systems (*in preparation*).*
- *UV-A and UV-C radiation induce the same pathway of D1 protein degradation as does UV-B (*in preparation*).*

This thesis is based on the following publications:

I Hideg, É., **Spetea, C.** and Vass, I. (1994) Singlet oxygen production in thylakoid membranes during photoinhibition as detected by EPR spectroscopy. *Photosynth. Res. 39*, 191-199.

II Hideg, É., **Spetea, C.** and Vass, I. (1994) Singlet oxygen and free radical production during acceptor- and donor-side-induced photoinhibition. Studies with spin trapping EPR spectroscopy. *Biochim. Biophys. Acta 1186*, 143-152.

III Hideg, É., **Spetea, C.** and Vass, I. (1995) Superoxide radicals are not the main promoters of acceptor-side-induced photoinhibitory damage in spinach thylakoids. *Photosynth. Res. 46*, 399-407.

IV **Spetea, C.**, Hideg, É. and Vass, I. (1996) Low pH accelerates light-induced degradation of the photosystem II reaction centre D1 protein by enhancing the probability of donor side mechanism of photoinhibition. Submitted to *FEBS Lett.*

V Friso, G., **Spetea, C.**, Giacometti, G.M., Vass, I. and Barbato, R. (1994) Degradation of Photosystem II reaction centre D1 protein induced by UVB radiation in isolated thylakoids. Identification and characterization of C- and N-terminal breakdown products. *Biochim. Biophys. Acta 1184*, 78-84.

VI **Spetea, C.**, Hideg, É. and Vass, I. (1995) Q_B-independent degradation of the reaction centre II D1 protein in UV-B irradiated thylakoid membranes. In: *Photosynthesis: from Light to Biosphere* (Mathis, P. Ed.) *Vol. IV*, pp. 219-222, Kluwer Academic, Dordrecht, The Netherlands.

VII **Spetea, C.**, Hideg, É. and Vass, I. (1996) The quinone electron acceptors are not the main sensitizers of UV-B induced protein damage in isolated photosystem II reaction centre- and core complexes. *Plant Sci.*, in press.

VIII Friso, G., Vass, I., **Spetea, C.**, Barber, J. and Barbato, R. (1995) UV-B induced degradation of the D1 protein in isolated reaction centres of Photosystem II. *Biochim. Biophys. Acta 1231*, 41-46.

These publications are referred to in the text by the above roman numerals.