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Minireview

Photoinhibition – a historical perspective*

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Abstract

Photoinhibition is a state of physiological stress that occurs in all oxygen evolving photosynthetic organisms exposed to light. The primary damage occurs within the reaction center of Photosystem II (PS II). While irreversible photoinduced damage to PS II occurs at all light intensities, the efficiency of photosynthetic electron transfer decreases markedly only when the rate of damage exceeds the rate of its repair, which requires *de novo* PS II protein synthesis. Photoinhibition has been studied for over a century using a large variety of biochemical, biophysical and genetic methodologies. The discovery of the light induced turnover of a protein, encoded by the plastid *psbA* gene (the D1 protein), later identified as one of the photochemical reaction center II proteins, has led to the elucidation of the underlying mechanism of photoinhibition and to a deeper understanding of the PS II 'life cycle.'

Abbreviations: bRC – bacterial reaction center; Chl – chlorophyll; DCIP – dichlorophenol-indophenol; DCMU – 3-(3,4, dichlorophenyl) 1,1- dimethylurea; EMBO – European Molecular Biology Organization; kDa – kilo Dalton, apparent; LMW – low molecular weight proteins of PS II; NASA – National Aeronautics Space Agency; pD1 precursor – carboxy-terminal unprocessed D1 protein; Pheo – the primary PS II electron acceptor; $Pheo^{\cdot-}$ – pheophytin anion radical; PI – photoinhibition; PS I, PS II – photosystem I and II respectively; Q_A – Q_B – $Q_A^{\cdot-}$ – $Q_B^{\cdot-}$ – the primary and secondary electron acceptors quinones of PS II in the oxidized and semiquinone radical form, respectively; RCII – photosystem II reaction center; S_2 – S_3 – the oxidation states of the manganese cluster of PS II donor side that has lost one or two electrons, respectively; Y_Z, $Y_Z^{\cdot-}$ – the tyrosine 161 of the D1 protein in the neutral and anion radical form, respectively

Introduction

Photoinhibition (PI) is a broad term that initially described the decline in photosynthetic viability of oxygen evolving photosynthetic organisms due to excessive illumination. The phenomenon was recognized more than 100 years ago, with the early work of Ewart (1896) who was amongst the first researchers to analyze photosynthesis and examine the effects of external factors on this intricate process (reviewed in Rabinowitch 1951). The importance of PI to the field of photosynthesis research could be judged by the number of publications devoted to its study, and by its prominent position as a major topic of heated, controversial discussion in meetings devoted to this topic since 1984, beginning with The EMBO (European Molecular Biology Organization) Workshop, on Pho-

* To Achim Trebst, for his interest, contribution, controversial discussions and appreciated support and friendship.

Figure 1a. Participants at the EMBO (European Molecular Biology Organization) Workshop on Photosystem II Dynamics held in Jerusalem, Israel, 1987. *First row (left to right):* Aviah Zilberstein, Franklin Callahan, Anastasios Melis, the late Philip Thornber, Itzhak Ohad and Marvin Edelman (organizers); Shmuel Malkin and Mordechai Avron are the last two in this row. *Second row, (left to right):* Eytan Harel, Beverly Green, Judith Brusslan, Joan Argyroudi-Akoyunoglou, Jan Anderson, Norio Murata, Ora Canaani, Jonathan Marder (behind him is Christa Critchley), Noam Adir, Diana Kirilovsky, Susan Golden, Elaine Tobin, Autar Mattoo (last). *Third row (left to right):* Aaron Kaplan (2nd), Joseph Hirschberg (3rd), Bruce M. Greenberg (6th, behind and on the left of N. Murata), Paul Mathis (7th, behind O Canaani), Gunnar Oquist (wearing glasses), Jonathan Gressel (behind N. Adir); John Bennett is behind and between S. Golden and E. Tobin; Achim Trebst and Gernot Renger (last two). *Last row (left to right):* Victor Gaba (behind and to the left of B. Greenberg), Wim Vermaas (beard and glasses), Hartmut Michel (behind C. Critchley), Judith St. John (right to J. Gressel), John Gray (behind S. Golden), Bertil Andersson and Jim Barber (last). Photo provided by Itzhak Ohad.

tosystem II Dynamics, Jerusalem, Israel, 1987 (see photograph of participants in Figure 1a).

Relatively early in the development of this research field, Bessel Kok and coworkers (Kok et al. 1966; see Kok's photograph in Jack Myers 2002) had identified PI as a phenomenon affecting specifically Photosystem II (PS II), and had characterized its major features: inactivation at high photon fluency of the electron flow and reduction of plastoquinone by PS II, phenomena related linearly to the intensity of the absorbed light. However, for many years the mechanism of this surprising discovery remained unclear until the necessary progress in understanding the mechanism of PS II electron flow, water oxidation, oxygen release and reversible electron flow were understood in more detail (reviewed by Debus 1992). Over the last three decades, progress in understanding PI has slowly moved from the descriptive and physiological, to its molecular aspects, and from *in vivo* experimentation to the use of *in vitro* systems including isolated thylakoid membranes and sub-membrane particles. Quite a number of reviews on the subject have been published with different focal points at their heart (Powels 1984; Barber and Anderson 1992; Prasil et al. 1992; Aro et al. 1993; Ohad et al. 1994; Keren and Ohad 1998). In this review we will summarize the development of the generally accepted views of PI, with emphasis on the historical perspective of the early phase of PI research in the mid-1980s to the mid-1990s, bridging the preceding phenomenological description of PI and our present (although still limited and not devoid of controversy) understanding of the molecular mechanism.

As in other energy and redox generating systems, photosynthesis is an intricate multi-step molecular phenomenon brought to its present complexity by a myriad of evolutionary steps. In other energy transducing systems, the primary driving force is of a chemical nature, which can be quantitatively measured, added or removed at the researcher's will. One could assume that different molecules used in and created via oxidative processes have specific binding sites within the system, that can be dissociated, analyzed as independent segments of the chain and then reconstituted as a whole, as demonstrated by the fast growing resolution of the mitochondrial oxidative phosphorylation process (Leverve and Fontaine 2001; Schagger 2002). Photosynthesis on the other hand is driven by light, which can potentially affect the entire photosynthetic system components and their interactions, antennas, reaction centers, accessory proteins, and induce secondary destructive and repair processes presently included under a general term, often ill defined: oxidative stress. Instead of a single, or at most a few 'binding' sites, light is absorbed by hundreds of pigment molecules, all working together to drive the process forward, or dissipate the excess absorbed energy by different routes. Thus the study of how light inhibits photosynthesis is complicated by its involvement in the activities of all steps performed by the photosynthetic system as well as the degradation and replacement of damaged components and re-assembly of functional units. To complicate matters further, evolution has created different species adapted to different environmental conditions and thus, responding differently to various levels of light intensity. These species-specific differences lead to a long-lasting lack of general conclusive evidence as to the primary lesion caused by light. The elucidation of the photosynthetic apparatus structure and function is intimately linked to the development of photoinhibition research. Historical aspects of the research of structure/function of the photosynthetic apparatus are described by other contributors (Clayton 2002; Renger, this issue). Barbara Demmig-Adams, (this issue), Govindjee (2002), and Govindjee and Seufferheld (2002) have discussed specific parts of the history of non-photochemical quenching of Chl fluorescence, the so-called photoprotection mechanism; thus, the above subject will not be elaborated here.

Photosynthesis at high light intensities

In the latter part of the 19th century, a number of researchers measured rates of photosynthetic activity as a function of light intensity. One of the earliest to identify that light behaves as a substrate, i.e., can achieve a level that saturates photosynthesis, was J. Reinke (Reinke 1883). He also concluded that saturating light levels are in the range of full sunlight intensity indicating that the photosynthetic system was prepared for the absorption of this maximal amount of light. It was A. J. Ewart (1896) who increased the light intensity beyond the saturation point and discovered that further addition of light induced a reduction of activity, to the point of total inhibition. Over the next decades, light curves were measured for a large variety of photosynthetic organisms, in most cases as a function of variation of a third parameter, such as partial pressure of CO_2, pH or the addition of various known chemical inhibitors of photosynthesis. These studies

have been reviewed in depth by Eugene Rabinowitch (1951). All of the studies at that period were carried out with whole organisms, and thus typically probed the intricate complexity of the entire system. Light was not alone in inducing a state of photoinhibition. It had been already shown by Otto Warburg (1919) that oxygen had an inhibitory effect on photosynthetic yield, and that the level of inhibition was higher under illumination at high intensities. Blackman and Smith (1911) suggested earlier that the effect of intense light is due to the inherent heating of the chloroplast and leaf, and that there is no inhibitory effect of photochemistry in itself. However, this turned out to be incorrect and there was already a suspicion that the inhibition is due to some form of direct, specific damage to the photosynthetic apparatus. In the early 1940s James Frank and C. Stacey French (1941) suggested that photo-oxidation, induced by either the presence of high levels of O_2 or high light intensity, damages the catalytic mechanism of photosynthesis. During the next decades, efforts to understand the PI process were focused on the attempt to identify the nature of the lesion(s) caused by light.

Already as early as the 1950s it was proposed that the damage caused by high light treatment was at the level of the primary photochemistry (Kok 1956). This occurred a number of years prior to the identification of the pigment-protein complexes involved in primary photochemistry and thus, it was difficult for many years to be any clearer on the nature of the actual mechanism and initial damaged site(s).

The search for the high light induced lesion

One of the difficulties in obtaining a general view of PI was due to both the physiological differences between the biological material used for experimentation, and in the actual definition of high light as an inducer of PI. Large variation in methods of the measurement of light intensity (number of photons/(area $\times$ time)), light quality (light intensity, wavelength) and presence of additional factors (temperature, atmospheric gas phase composition, presence of ultra-violet light, addition of inhibitors of protein synthesis) made comparisons difficult and impeded progress on identification of the major lesion. However, ultimately the conclusions reached by most of the community of researchers involved in PI study were: i). The major lesion leading to PI is generated at a rate related to the light intensity and *in vivo* it is efficiently repaired at lower light intensities. Thus PI is observed either under high light conditions when the repair mechanism(s) have reached maximum capacity, or at lower light intensities when an additional external factor inhibits repair. ii). Extended high light exposure induces a plethora of secondary effects, each of which contributes to the measured decrease in photosynthetic capability.

S. Powels (1984) reviewed the then current knowledge on PI covering primarily the realm of physiology, with special emphasis on the differences between sun and shade plants, and the resulting PI in the presence of additional forms of stress factors. In the studies of that period, measurements were made on both whole electron chain transfer activity (such as oxygen evolution *in vivo*) and on partial reactions (such as chlorophyll (Chl) fluorescence induction in isolated thylakoid membranes in the presence of DCMU (3-(3,4,dichlorophenyl) 1,1-dimethylurea) which probes only Photosystem II (PS II) activity). The action spectrum of PI was shown to be similar to that of photosynthesis, indicating that it was photosynthetic activity itself which causes the damage, and not just light absorption (Jones and Kok 1966). It was clear that inhibition of photosynthesis could occur via a number of pathways, although it was not always easy to make general statements on phenomena measured in a large variety of organisms and conditions. Furthermore, it was shown by some groups (Satoh 1970; Inoue et al. 1986; Sonoike and Terashima 1994) that PS I activity could be diminished under PI conditions, although it could generally be stated that PS I inhibition was significantly limited as compared to that of PS II under the same conditions and was perhaps of a secondary nature. Isolation of PS I and resolution of its major components was achieved already during the middle of the 1970s (Bengis and Nelson 1975, 1977; see also Nelson 2002). As shown recently, the mechanism of PS I photoinactivation is due to light and low temperature effects and a slow replacement process of its PsaB subunit (Sonoike 1996). Indeed, other 'secondary' forms of PI were identified, usually by the presence of an additional form of stress. On the other hand, it appeared to many researchers that PS II was heavily damaged by PI conditions, even without added stress. The loss of PS II activity could be shown by a variety of techniques, all pointing to a loss of PS II electron transport capabilities. From the cumulative results of the groups in the field from the 1950s to the beginning of the 1980s it could be hypothesized that PS II was in the least the major site of PI

(Powels 1984; Kyle 1987) and that the actual point(s) of damage could now be identified.

Characterization of photoinhibition at the molecular level

Since the early 1980s the biological sciences have seen an explosion of technical breakthroughs. Biochemistry and biophysics continued to make consistent strides at improving the quality of analytical methods and tools, and in the study of photosynthesis this led to high resolution of the thylakoid membrane polypeptides by sodium dodecyl sulphate polyacrylamide gel electrophoresis (Delepelaire and Chua 1981; Chua and Bennoun 1975), better isolation procedures of the different thylakoid membrane complexes (Åkerlund et al. 1976; Berthold et al. 1981; Thornber et al. 1984; Peter and Thornber 1991), and to more accurate measurements of their biophysical properties using spectroscopy and fluorescence techniques (Joliot and Joliot 1984; Govindjee et al. 1990; Witt 1991), EPR techniques (Babcock et al. 1989; Petersen et al. 1990; Hideg et al. 1994 a, b) and thermoluminescence (Inoue and Shibata 1982; DeVault et al. 1983; Demeter and Govindjee 1989), to mention only a few. The advances in molecular biology allowed manipulation of the biological material in a way previously not possible (Kirilovsky et al. 1989; Przibilla et al. 1991; Erickson and Rochaix 1992; Ohad and Hirschberg 1992; Pakrasi and Vermaas 1992; Clarke et al. 1993; Mäenpää et al. 1993; Mulo et al. 1998). The emerging knowledge of the sequences of the proteins involved in photosynthesis allowed the use of site-directed mutagenesis of potentially important amino acid residues. Structural biology finally began to have an impact on photosynthesis research through the determination of the structures of the first membrane protein complexes, bringing with it a molecular view of photosynthesis only dreamed of before. These tools were slowly brought into the study of PI, as the molecular origins of inhibition were sought out.

PS II heterogeneity

Starting from the early 1960s, the photosynthetic membranes of plants and algae and their development were extensively studied using various techniques. The intricate organization and structure of the photosynthetic membrane was studied by electron microscopy in the green alga *Scenedesmus* (Weier et al. 1966). Structural changes in the membrane organization during thylakoid biogenesis were studied as well in *Euglena* (Ben-Shaul et al. 1964) and the green alga *Chlamydomonas* (Ohad et al. 1967). Separation of submembrane particles by density gradient centrifugation and phase partition allowed the tentative identification of the relative positions of the different photosynthetic complexes and their activity in different membrane domains. The chloroplast membrane heterogeneity was intensively studied (Åkerlund et al. 1976; Andersson et al. 1976; Andersson and Anderson 1980; Andersson 1982; Albertsson et al. 1990; Albertsson 1995; see also Albertsson 2001 and Anderson 2002) (see Figure 1b for a photograph of Andersson). These findings pointed out the possibility that different membrane components may be subject to lateral mobility within the membrane plane. Electron microscopy in combination with freeze fracturing of the specimen to be observed (Staehelin 1986; see Figure 1c for a photograph of Staehelin) demonstrated that the distribution of membrane protein complexes is heterogeneous. Soon it was realized that PS I and the ATP synthase were found almost exclusively in the unappressed stroma lamella regions, while a large proportion of PS II and LHC II could be found in the appressed grana lamella (see Anderson 2002). One of the most striking results of these studies was the identification of multiple types of PS II. The complexes located in the grana lamellae were functionally different from those found in the stroma lamella. As opposed to the grana located α centers, PS II in the stroma lamella (termed PS IIβ) were not active in electron transfer to plastoquinone and had a limited antenna size (Anderson and Melis 1983, see Figure 1a for a group photograph that includes Melis and Anderson). The existence of different forms of PS II gave rise to a number of interpretations, including the possibility that PS II in the grana lamellae perform cyclic electron flow and are disconnected from the linear electron transfer pathway that occurs in the stroma lamellae (Arnon and Tang 1989). The realization that the photosystems, antennae, cytochrome b_6f complex and the proton ATP synthase are not randomly distributed in the thylakoid membranes prompted experiments designed to isolate sub-membrane fractions and characterize the activity and composition of their protein/pigments complexes. The heterogeneity of PS II correlated to the presence of photoinhibited PS II (Neal and Melis 1991). Furthermore, after exposure to high light intensity, the β centers were less susceptible to photoinhibition and did not recover activity as compared to the α centers (van Wijk et al. 1993).

Figure 1b. Bertil Andersson and Torill Hundal (during a visit to Jerusalem) and Itzhak Ohad. Photo provided by Susana Shochat.

Figure 1c. Joseph Hirschberg (left), Itzhak Ohad and Andrew Staehelin. Photo provided by David Kyle.

Figure 1d and e. Marvin Edelman (left) and Itzhak Ohad (right). Photos provided by M. Edelman and I. Ohad, respectively.

Figure 1f. David Kyle (left) with Kit Steinback and Charles J. Arntzen. Photo provided by D. Kyle.

Figure 1g. Marvin Edelman's group: F.E. Callahan, M. Edelmaan, Autar Mattoo, Victor Gaba, Jonathan Marder and B.M. Greenberg. Photo provided by M. Edelman.

Enter the D1 proteins

Final identification of chlorophyll binding proteins that served as light-harvesting antennae preceded that of the proteins forming the photochemical reaction centers. Studies of the biogenesis of thylakoid membranes developed in parallel to those of the development of the photosystem activities (Ohad et al. 1967; Wallach et al. 1972; Eytan et al. 1974; Bar-Nun et al. 1977). Synthesis of light-harvesting antennae could be dissociated from that of the PS II electron transfer complex (reviewed in Ohad and Drews 1982). The identification of the various Chl-protein complexes acting as antennae has progressed as well (Bassi et al. 1987). In the process of thylakoid membrane development studies it was found that trypsin cleavage of a protein of about 30 kDa exposed on the matrix side of the thylakoid membrane abolishes the inhibition of PS II electron flow to dichlorophenol indophenol (DCIP) by DCMU but not to ferricyanide (Regitz and Ohad 1976). A similar finding was independently reported by Gernot Renger (1976; see Figure 1a for a photograph that includes Renger). However at that time, no exact function could be ascribed to this protein.

Renger proposed that it could serve as a molecular 'shield,' protecting in some way the other proteins of the reaction center (see Renger, this issue). Progress in the research of bacterial photosynthesis had established much earlier (Clayton and Smith 1960) that the photochemistry of purple non-sulfur bacteria is performed by a protein complex containing only three proteins, M, L, and H. Further, their primary amino acid sequence had been established (Williams et al. 1983, 1984) and the structure of the complex was resolved by X-ray crystallography (Deisenhofer et al. 1984, 1985; Allen et al. 1987; see Clayton 2002; Jim Allen, to be discussed in Part 3 of these history issues). Although a clear similarity was already evident between the primary charge separations, the reduction of pheophytin and of quinone in the anoxygenic bacterial reaction center (bRC) and that of the oxygenic PS II (Michel and Deisenhofer 1988), a possible similarity at the level of protein composition of the two photochemical reaction centers was not even considered. The possibility that Chl-proteins identified by non-denaturing gel electrophoresis such as CP43 and CP47 (Chl protein complex of 43 kDa and 47 kDa, respectively) were essential for the formation of active PS II was supported by the use of a temperature sensitive mutant of the unicellular green alga *Chlamydomonas* (Chua and Bennoun 1975) and for a while, CP47 was considered as a candidate for being the component involved in the charge separation process of PS II photochemical reaction center (see, e.g., Camm and Green 1983). The study of protein synthesis by radioactive labeling in isolated chloroplasts, aimed at understanding the organelle biogenesis, revealed light-dependent synthesis of several thylakoid protein bands as resolved by SDS-PAGE, among them a major one termed the 'peak D' (Eagelsham and Ellis 1974). Pulse-chase studies of protein synthesis *in vivo* using the duckweed *Spirodella*, led Marvin Edelman (see Figure 1d for a photograph) and coworkers to the discovery of a fast turning-over thylakoid membrane protein of 32.5 kDa and the characterization of this process (Edelman and Reisfeld 1978; Reisfeld et al. 1982). The 'shield' protein was identified as the rapidly turning over 32.5 kDa protein (Mattoo et al. 1981). At the time that this idea was proposed, the fast turning over protein was thought to have a peripheral position in PS II, and could thus easily be dispensed of and replaced upon onset of damage. However, a number of experimental surprises showed that the turnover of this protein involves a much more complicated process.

The first encounter by one of us (Itzhak Ohad, Figure 1e) with the topic of photoinhibition occurred during a Sabbatical period spent in 1983 in the laboratory of Charles Arntzen, then Director of the Plant Research Laboratory (PRL), at the Michigan State University at East Lansing. A Sabbatical is well spent when you join an ongoing project and team with a group of young researchers working on a subject that is not familiar to you and could provide you with knowledge and technology that may be useful when you return home. This ensures excitement, fast progress of the work and it establishes lasting relations with future scientists who will lead the field in their turn. Charlie Arntzen, who was always attracted by topics that have also relevance from the point of view of application ('What do I tell the farmer about this research?,' he used to say), suggested to me (I. O.) to join David Kyle and Kit Steinback (see Figure 1f for a photograph; see also photographs of Arntzen and Steinback in Allen 2002) who were beginning to study the phenomenon of photoinhibition, then considered already to be relevant to plant productivity. Using pea plants, they asked basic questions concerning the kinetics, light intensities and standardization of measurements of photosynthetic activity aimed at understanding the underlying mechanisms. I (I.O.) had practically no experience with higher plants; my (I.O.) 'organism of choice' at that time was *Chlamydomonas reinhardtii* and my work was centered since the middle 1960s on the greening process and biogenesis of the thylakoid membranes.

The facility of experimental work with *Chlamydomonas* cells, and the advantage of using an oxygenic eukaryote that can be handled as a microorganism (Ohad et al. 1967), as was also the case with aquatic higher plant, *Spirodella*, then used by the Edelman group (see Figure 1g for a photograph) prompted us to use these cells for the study of photoinhibition *in vivo.* The aim was to understand the mechanism of the light induced loss of PS II activity and most important, the recovery process, trying to establish if protein synthesis is required for the recovery of oxygen evolution and if so, identify the protein(s) involved. This work established that *in vivo*, PI is due to loss of plastoquinone (PQ) reduction by PS II, while the rest of electron transfer chain remains unaffected (Kyle et al. 1984). The surprise came when it was discovered that recovery of PS II activity occurring within a few hours, in the absence of cell division, required synthesis of a plastid translated protein of about 32 kDa (Ohad et al. 1984). Partial repair of PS II activity

Figure 1h. Participants at a Satellite meeting of the XI Congress on Photosynthesis in Budapest, Hungary, 1998. The satellite meeting was dedicated to Photosystem II and organized by Imre Vass at Szeged, Hungarian National Academy of Science, Szeged, Hungary. *First row (left to right):* Prassana Mohanty, Barry Osmond, Heinrich Krause, Kintake Sonoike, Manuel Pinto, Marcal Jansen, Linda Franklin, A. N. Mishra, Krishna Niyogi, Dejan Markovic, Alia, Josef Komenda, Imre Vass, Anne-Lise Etienne, Itzak Ohad, Diana Kirilovsky, Jan Anderson, Anthony Larkum, Roberto Barbato, Mitsue Miyao-Tokutomi, Suzanna Sochat, Torill Hundall, Sohiela Mackerness. *Second row (left to right):* Peter Nixon, Eva-Mari Aro, Éva Hideg, Francesco Ghetti, Borbála Bálo, Giovanni Checcucci, Schulz, Alejandro Riquelme, Maria Bianchetti, Tzvetlina Markova, Katia Marinova Georgieva, Cornelia Spetea, Esa Tyystjärvi, Konstantin Neverov, Gunnar Öquist, Olga Koroleva, Fatbardha Babani, Arja Pennanen. *Third row (left to right):* Thomas Vogelmann, Bertil Andersson, James Barber, Christa Critchley, Olimpio Montero, Youn-il Park, Gyula Váradi, László Makrai, Peter Jahns, Mira Busheva, Anja Kriger-Liszkay, Tsonko Tsonev, Sonja Veljovic-Jovanovic, Carola Bösinger, Ondrej Prasil, Lesleigh Force, Maria Rova, Martin Tritlek, Kozi Asada, Zoltán Máté, Julia Tandori, Zsolt Csintalan, Alberto Vianelli, András Viczián, Jun ichi Mano, Belinda Morrison, Naoki Mizusawa, László Sass, Ursula Lütz-Meinl, Cornelius Lütz. Photo provided by Imre Vass.

occurred also in the absence of cytosolic protein synthesis. Furthermore, recovery of activity could occur at least partially in darkness, interpreted at that time, as indicating that sustained *de novo* Chl synthesis is not necessary (Ohad et al. 1984). At the same period of time, Joseph Hirschberg (see Figures 1a and c for photographs) was spending a postdoctoral study period in Lee McIntosh's laboratory also at the PRL in East Lansing. Using an *Amaranthus* herbicide resistant mutant, they established that a single amino acid replacement in a plastid encoded protein of about 32 kDa ascribes resistance to herbicides of triazine, urea and phenolic type (such as atrazine, DCMU and ioxinyl, respectively), known to inhibit electron flow from the reduced quinone acceptor of the PS II complex Q_B to the plastoquinone pool. Thus, it was established that this protein is involved in the function of the secondary quinone acceptor, Q_B site (Hirschberg and McIntosh 1983) and acts as the 'the Q_B protein.' Work by the late Kit Steinback concurred and it became clear that the protein damaged and needed for the repair of PS II activity was the earlier identified Q_B, herbicide binding protein (Pfister et al. 1981; Mattoo et al. 1981, see Figure 1g for a photograph of Mattoo; Satoh et al. 1983). These results could explain the nature of the fast turning-over protein of 32.5 kDa. Turnover of this protein was thus related to the inactivation and recovery of the acceptor side of PS II activity. Based on the partial homology between the L protein of the bacterial reaction center (Hearst and Sauer 1984) and that of a thylakoid protein that migrated as a diffuse band in SDS-PAGE, termed the D1 protein, it became evident that D1 protein is the same as the 32.5 kDa fast turning over protein, the Q_B protein, and the thylakoid 'peak D' protein earlier studied by R. J. Ellis and coworkers found to be synthesized in isolated chloroplasts only when exposed to light (Eaglesham and Ellis 1974).

The proposal that degradation and *de novo* synthesis of this protein are responsible for the light-induced PI and recovery of activity (Kyle et al. 1984; Ohad et al. 1984) met with approval by many as well as strong opposition (Christa Critchley 1988, see Figures 1a and h for group photographs) arguing that charge separation must be the first step to be inactivated during PI. Thermoluminescence measurements of PS II charge recombination combined with electron paramagnetic resonance (EPR) measurements of PS II signals indicated that the initial stages of PI are characterized by alteration of the Q_B site activity localized in the D1 protein and not to the loss of charge separation (Ohad et al. 1988). The concept that the photoinactivation of PS II in thylakoids or *in vivo* is initiated by loss of acceptor side activity prior to the loss of charge separation was supported and extended by findings from other laboratories (Nedbal et al., 1990; Setlik et al. 1990, see Figure 2a for a photograph of Setlik; Andersson et al. 1992; Vass et al. 1992). While the experimental results reported in the initial publications using *Chlamydomonas* (Kyle et al. 1984; Ohad et al. 1984) remain valid until today, the interpretation of the data offered at that time was not completely correct. It soon became evident that the light-induced loss of PS

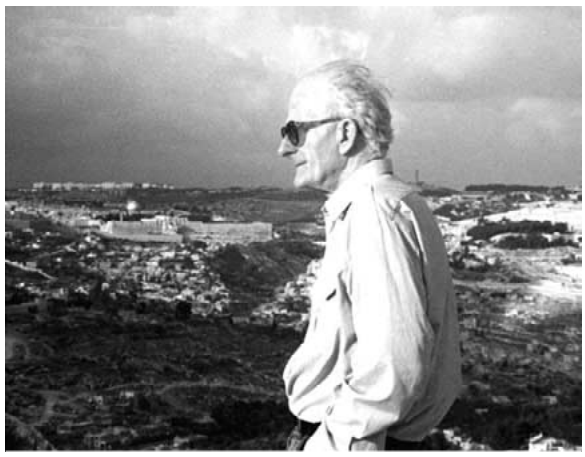

Figure 2a. Ivan Setlik, on a visit to Jerusalem 1989. Photo provided by I. Ohad.

Figure 2b. Eva-Mari Aro, during a visit to Israel, picking spinach in Galilee for preparation of light-harvesting complex, LHC II, and protein kinase. Photo provided by I. Ohad.

Figure 2c. Achim Trebst. Photo provided by A. Trebst.

Figure 2d. Itzhak Ohad group: (left to right) Huashi Gong, Itzhak Ohad, Nir Keren, Gideon Mazor, Susana Shochat, and last, Shlomit Tal. Photo provided by S. Shochat.

Figure 2e. Kimiyuki Satoh (left) and David Kyle. Photo provided by D. Kyle.

Figure 2f. James (Jim) Barber. Photo provided by J. Barber.

Figure 2g. Gadi Schuster. Photo provided by G. Schuster.

II activity preceded the degradation of the D1 protein and the degradation of the protein is the result of light induced damage to PS II and not the primary cause of the phenomenon (reviewed in Prasil et al. 1992; Aro et al. 1993, see Figure 2b for a photograph of Aro). While attempts were made to identify oxidation of specific amino acids in the D1 protein following light exposure of thylakoids, the exact molecular characterization of this assumed oxidative damage remains unsolved until today. However, it was established that photoinhibition was accompanied by the exposure of a specific site of the D1 protein to proteolytic cleavage. This site was the same as the cleavage site of the fast turning over 32.5 kDa protein (Greenberg et al. 1987, see Figure 1g for a photograph of Greenberg) identi-

fied much before the relevance of this observation to PI was realized. The idea that the D1 protein turnover is due to, or causes, photoinhibition was still not accepted by many of those studying this phenomenon. In an excellent review of the dynamics of the D1 protein, the possibility that the turnover of this protein could be related to photoinhibition was not considered (Mattoo et al. 1989). The initial cleavage site of the D1 protein was demonstrated to be exposed on the matrix side of the thylakoid membrane (Greenberg et al. 1987). The location of this site was ascribed to the DE helix of the D1 protein (De Las Rivas et al. 1992) assumed to be a membrane parallel helix interconnecting the transmembrane D and E helices on the stromal thylakoid membrane face. This was in agreement with the suggestion that the light induced damage may be related to the exposure of the site to the protease (Trebst 1991, see Figure 2c for a photograph of Trebst). The search for a reason for the rapid degradation of the D1 protein other than that of PS II photoinhibition led Edelman and co-workers to propose the 'Pest hypothesis' whereby the rapid turnover of the D1 protein was a natural consequence of the presence of *PEST*-like sequence (Mattoo et al. 1989). The Pest sequence includes a P (proline), E (glutamic acid), S (serine) and T (threonine). This sequence is common in unrelated proteins undergoing rapid degradation in non-photosynthetic tissues. The PEST-like sequences are located close to the cleavage site of D1 proteins from various sources sequenced at the time. However alteration of this sequence by site directed mutagenesis (Tyystjärvi et al. 1994; Mulo et al. 1998) and analysis of the location of the initial cleavage site (Barbato et al. 1991) did not provide conclusive support for this hypothesis. For quite some time, the question of the primary damage, its mechanism and its relation to the degradation of the D1 protein has become the major subject of research concerning the elucidation of the PI phenomenon.

The initial work summarized above established the basic features of the PI and recovery processes, the latter being often referred to also as the 'Repair cycle': Light induced damage, occurring at light intensities higher than those needed to saturate photosynthetic electron flow, induces irreversible loss of PS II activity that can be recovered only following degradation of the D1 protein and its replacement by a *de novo* synthesized molecule, the translation product of the plastid *psbA* gene. The first direct evidence that the amount of the *de novo* synthesized D1 protein is directly related to that of protein degraded was provided by work carried out by Susana Shochat in the Jerusalem laboratory (Ohad et al. 1988, see Figure 2d for a photograph of the Ohad group).

As mentioned above, the bacterial RC structure at 3 Å resolution was determined by Deisenhofer and coworkers (1984, 1985). The relevance of these findings to the structure of PS II RC (Michel and Diesenhofer 1986, 1988) changed our views on PS II photochemistry and of photosynthesis and reaction centers in general. At about that time, DNA sequences of the D1 and D2 proteins from a large variety of plant and algal species had already become available; it was shown that these two proteins were partially homologous to the L and M core proteins of bRC. The idea that the D1 protein was actually part of the PS II RC was first suggested by Achim Trebst (1986). Nixon et al. (1986) showed that the D1 and D2 proteins were present in the core of PS II. At the VII International Congress on Photosynthesis at Rhode Island, the order of presentations in one session was changed just before its beginning and to the surprise of all participants an unscheduled lecture was presented: Kimiyuki Satoh (see Figure 2e for his photograph) reported the isolation of the PS II reaction center. This was the first demonstration that a protein/Chl complex consisting of D1 and D2 heterodimer proteins and cytochrome b559 can perform the light induced charge separation process (Nanba and Satoh 1987; see Satoh, this issue). This was a crucial finding to be followed and expanded in many laboratories (see, e.g., Barber et al. 1987, see Figure 2f for a photograph of Barber; Michael Wasielewski et al. 1989, see Seibert and Wasielewski, this issue, for photographs; Scott Greenfield et al. 1997; Jan Dekker and Rienk van Grondelle 2000). This of course made the concept of the turnover of PS II reaction center protein all the more difficult. On the other hand, it immediately suggested a reason behind the turnover. The major difference between the bRC, that is not subject to light induced inactivation and degradation of its subunits, and PS II RC is the much higher oxidizing potential needed to abstract electrons from water in PS II. This potential leads to the formation of a number of transiently short-lived radical species on both the reducing side ($Pheo^{\cdot -}$, $Q_A{}^{\cdot -}{}_{\cdot}$ $Q_B{}^{\cdot -}$) as well as the oxidizing side of PS II (Mn^{n+} $Y_z{}^{\cdot -}$) (reviewed in Debus 1992). These radicals could oxidize the RC II protein matrix either directly or by the production of singlet oxygen ($^1O_2$) or hydroxyl radicals (Macpherson et al. 1993; Hideg et al. 1994a; Telfer et al. 1994). This was in line with previous proposals by a number of research groups

that indeed the D1 protein goes through a process of radical-induced specific damage, which leads to the shutting down of PS II activity (protecting the rest of the RC). The degradation/turnover resulted from the modification of the D1 protein (reviewed in Prasil et al. 1992).

Experiments carried out *in vitro* using intact thylakoids demonstrated that the proteolytic activity degrading rather specifically the D1 protein in high-light exposed membranes can be ascribed to a membrane bound protease(s) (Ohad et al. 1985; Reisman and Ohad 1986). Attempts to further dissect the system and use isolated PS II cores or the isolated D1/D2 heterodimer to probe for the D1 proteolysis resulted in the degradation of the protein (Virgin et al. 1990), as well as formation of aggregates, possibly adducts of D1 protein and other PS II components (Barbato et al. 1992) that presented some difficulties in the interpretation of the results. The search for the protease tentatively identified as the Chl *a*/protein complex CP43 (Salter et al. 1992) and even the D1 protein itself (Shipton and Barber 1994) has mobilized efforts of several laboratories and has culminated only recently by the identification of the FtsH and DegP type proteases as the real players in the game (Adam 2000; Lindahl et al. 2000; Haussuhl et al. 2001). Since oxygen involvement in the D1 protein turnover was implied, experiments were addressed to test the effect of anaerobiosis on the degradation of the D1 protein. The protein was not degraded if thylakoids were exposed to the light under N_2 atmosphere while photo-inactivation of PS II occurred (Arntz and Trebst 1986). However this process reversed upon further incubation of the membranes after air admittance in low light in absence of *de novo* synthesis of the D1 protein (Hundal et al. 1990, see Figure 1b for a photograph of Hundal). It is interesting to note that photoinactivation of PS II measured as oxygen evolution in isolated thylakoids under anaerobic conditions was first reported as part of a NASA (National Aeronautics Space Agency) supported program led by Jack Myers already in 1962 (see Trebst 1962). The D1 proteolysis turns out to be a complicated multistep process involving participation of several proteases, requiring GTP and ATP (Spetea et al. 2000). It is well established that redox-controlled reversible phosphorylation of PS II-associated proteins regulates energy transfer and the process of state transition (reviewed in Gal et al. 1997; Keren and Ohad 1998; Allen 2002). Furthermore, the degradation of the D1 protein was shown to be regulated by the protein phosphorylation/dephosphorylation state (Elich et al. 1992; Koivuniemi et al. 1995; Rintamäki et al. 1995, 1996; Ebbert and Godde 1996).

Photoinhibition and D1 protein turnover

The relation between D1 protein turnover and PI, first recognized by David Kyle, one of us (I.O.) and Charles Arntzen in 1984, was quickly followed by a number of other groups who concurred with their results (reviewed in Prasil et al. 1992; Ohad et al. 1994). Throughout the 1980s and the 1990s, major attempts were made to correlate the rate of turnover of the D1 protein and the level of PI, at the level of whole plants (Godde 1992; Park et al. 1996; Anderson et al. 1998), algae (Schuster et al. 1988, see Figure 2g for a photograph of Schuster; Gong and Ohad 1991, see Figure 2d for a photograph of Gong) or cyanobacteria (Ohad et al. 1990; Komenda and Masojidek 1995). As mentioned above, the observed correlation between these events *in vivo* led for the first time to the study of PI and the D1 protein degradation *in vitro* systems. In these experiments, performed on both higher plant membranes (Ohad et al. 1985) and algal thylakoids (Reisman and Ohad 1986), D1 protein degradation/turnover related to PI was measured by radioactive pulse chase experiments analyzed by autoradiography, and the concomitant measurement of PS II activities: oxygen evolution, Chl fluorescence induction and thermoluminescence (see Vass, this issue, and Figure 1h for a group photograph that includes Vass). The latter technique that could detect charge separation and recombination *in vivo* was first used to probe PI by I.O. during a period of work under the guidance of Yorinao Inoue and Hiroyuki Koike at the Riken Research laboratory, Wako, Saitama, Japan (Ohad et al. 1988). The results obtained in these experiments showed that there was a direct correlation between the losses of pre-labeled D1 protein, and the loss of PS II activity when *Chlamydomonas* cells are exposed to high light. Furthermore, PI correlated with the loss of acceptor-side activity of PS II and plastoquinone occupancy of the Q_B site.

With the development of the first anti-D1 protein antibodies, the actual amount of D1 protein could be quantified at different times following exposure to different light intensities. The coupling of the radioactive pulse-chase experiments (Mattoo et al. 1984) with the immunodetection methods showed conclusively that D1 protein turnover, that occurs at all light intensities,

Figure 3. Hagit Zer (left) and Noam Adir (right). Photos provided by H. Zer and N. Adir, respectively.

is a natural part of PS II activity. However due to its rapid replacement, the protein is not depleted unless its synthesis is rate limiting in reassembling functional PS II complexes.

The occupancy of the Q_B site by plastoquinone or other Q_B ligands may regulate the damaged protein degradation as previously proposed based on analysis of D1 degradation in cytochrome b_6f-deficient *Chlamydomonas reinhardtii* mutants (Gong and Ohad 1991, see Figure 2d for a photograph of Gong). Over-reduction of the plastoquinone pool in mutants deficient in cytochrome b_6f, PS I or plastocyanin retarded degradation of the D1 protein during light exposure and allowed its degradation upon transfer of the cells to low light allowing oxidation of the PQH_2 pool (Zer et al. 1994, see Figure 3 for a photograph of Zer). Furthermore, inhibition of PS II activity by herbicides partially prevented the degradation of the D1 protein (Zer and Ohad 1995). This was in line with the fact that the first cleavage site (the DE loop) was located in the Q_B site region. However, while the degradation of the protein induced by light exposure was retarded by binding of DCMU at the Q_B site, it turned out that generation of $^1O_2$ and inactivation of PS II RC are actually enhanced by changes in the redox potential of $Q_A^{\cdot -}$ upon binding of this herbicide at the Q_B site (Rutherford and Krieger-Liszkay 2001). This explains why quenchers of free radicals, that were used to test their effect on the turnover of the 32.5 kDa protein, lowered the rate of D1 protein degradation (Sopory et al. 1990) in line with the explanation that $^1O_2$ induces the damage leading to the PS II protein degradation. The role of singlet oxygen in this process is now reiterated by new findings (see Trebst et al. 2002).

Uncovering the D1 damage/repair mechanisms became one of the major efforts in PI study (Adir et al. 1990, see Figure 3 for a photograph of Adir; Ohad et al. 1990; Shochat et al. 1990; Keren et al. 1995, 1997, see Figure 2d for a photograph of Keren and Shochat).

Measurable onset of electron flow inhibition occurred only at elevated light intensities and thus, apparently did not correlate with the degradation/turnover of the D1 protein, found to occur even at low light intensities. This served as an argument against the hypothesis that D1 protein degradation is related to PI. However, the requirement for *de novo* D1 protein synthesis for recovery of photosynthetic electron flow offered an explanation for this discrepancy. Thus, the onset of measurable PI is not due to the actual damage and degradation of the D1 protein, rather it occurs due to limitations of the D1 replacement system, as first proposed by Kyle and Ohad (1986) (see also Leitsch et al. 1994; Melis 1999; Allakhverdiev et al. 2002). Furthermore, common sense dictated that back electron flow and charge recombination occur mostly at saturating light intensities and thus it is incompatible with the D1 protein turnover that occurs also at low light intensities. Yet based on preliminary results, Ohad and coworkers argued that back electron flow, charge recombination and generation of $^1O_2$ can occur even at very low light intensities. An 'integrated theory' proposing that the same mechanism is responsible for the D1 degradation at all light intensities, that met with approval by many as well as disbelief by some, was presented at the Harden Conference dedicated to the study of photoinhibition organized by Neil Baker and John Bowyer in 1994 (see in Figure 3c for a photograph of participants). This theory ascribed degradation of D1 protein at low light intensities as being due to recombination of $Q_B^{\cdot -}/S_{2,3}$ donor side states when the slow rate of PS II excitation allows a long life time of this charge separated pair (Ohad et al. 1994). Studies carried by Nir Keren and Huashi Gong using *Chlamydomonas* cells proved that actually low light intensities induce degradation of the D1 protein far below the saturation level and are even more effective in terms of degraded D1 protein/light absorbed, than higher light intensities (Keren et al. 1995, 1997). This phenomenon could be explained on the basis of measurements of PI as a function of the number of single turnover flashes given as single or consecutive pairs at increasing dark intervals aimed at testing the effect of the life time of $Q_B^{\cdot -}$ semiquinone that increases as the excitation frequency of PS II decreases. Exposure of *Chlamydomonas reinhardtii* cells to single saturating light flashes, given at increasing time intervals,

Figure 3c. Participants at the Harden Conference 1993, dedicated to 'Photoinhibition in plants,' organized by Neil R. Baker and John R. Bowyer. *First row (from left to right and in numerical order):* Nir Keren (1st), Hagit Zer (2nd), Diana Kirilovsky (3rd) Itzhak Ohad (4th), Doris Godde (5th), John Bowyer (11th) Jonathan Marder (12th), Margrit Broza-Robol (14th), Ivona Adamska (16th) (Caroline Jegerschöld is behind I. Adamska), Mitsue Miyao (18th), Imre Vass (19th); (Eva Rintamaki and Heinerich Krause can be seen behind M. Miyao); Alaka Srivastava (20th); Kozi Asada is behind Alaka. *Second row (left to right):* A. Salter (3rd) Stenbjorn Styring (4th), Bertil Andersson is in the second row (above and between M. Broza and I. Adamska); John Whithmarsh is one or two rows above, and second from B. Andersson. *Third row:* Don Ort (4th, left and above Styring), last on the right side is Jim Barber (above and to the right of Vass). Photo provided by Jim Barber.

Figure 3d. A photograph of participants at a Summer School in Turku (Finland) organized by Eva-Mari Aro. Here, a game of 'electron transport in photosynthesis' was organized by Govindjee (first row, first on the left). Different 'components' of Photosystem (PS) II and PS I transferred electrons and protons (balloons) following a 'flash' (scream, by Esa Tyystjärvi, last row, left of Itzhak Ohad (center), wearing dark glasses). The components are not organized as played during the game. Itzhak Ohad played the role of 'Pheophytin', and Eva-Mari Aro to his right was the reaction center of PS II, 'P_{680}'; primary charge separation was induced by the 'light flash'; electrons went from excited P680* (Aro) to Pheo (Ohad); the manganese cluster (to her right) provided the lost electron to her as she had turned into $P_{680}{}^{+}$; the electron on pheophytin (Ohad) was passed to Q_A (Esa); charge recombination between reduced pheophytin and oxidized P_{680} leading to light emission had little chance to occur in the game. Photo and text provided by Govindjee.

generates $Q_B{}^{\cdot -}$ followed by charge recombination. As a result, massive loss of the D1 protein occurs after only 1440 single light flashes given at 240 s intervals. The number of charge recombination events in this case represents only a fraction of a percent of the number of charge separations and electrons transferred to plastoquinone during excitation of thylakoid membranes for a few minutes. However, a similar number of consecutive pairs of flashes (0.5 s apart) delivered at the same interval, which doubly reduced Q_B (followed by protonation and plastoquinol release), almost completely abolished this effect. Thus, increasing the chance of charge recombination at low excitation rates is responsible for the D1 protein degradation and not the total number of PS II excitations. Cells exposed to the above treatments in the absence of protein synthesis inhibitors, allowing degradation and replacement of the D1 protein, showed no net loss of D1 protein or manifestation of PI. The important conclusion of this work was that turnover of the D1 protein observed at low light intensities (that initially could not be ascribed to over-excitation of PS II) was actually due to charge recombination promoted at low rates of PS II excitation. While the proposed theory ascribing the turnover of the D1 protein at all light intensities as being due to charge recombination and generation of $^1O_2$ gained support, controversy persisted. The concept was challenged on the basis of measurements of the initial PI velocity and calculations of the rate constants of PI versus light intensity (Tyystjärvi and Aro 1996; see Figure 3d for a group photograph that includes Tyystjärvi and Aro). On the basis of such measurements, it was argued that PI is not related to electron flow and its regulation but rather to the photosystems' Chl excitation *per se.* Other explanations such as the possibility that weakly coupled Chl/proteins may generate singlet oxygen have been proposed (Santabarbara et al. 2002). A 'unifying model' for the photoinhibition *in vivo* ascribing photoinactivation of PS II to damage induced by $P_{680}{}^{\cdot +}$ cation radical and not being related to generation of singlet oxygen was proposed by Jan Anderson and coworkers (1998). However the involvement of back reactions and charge recombination in the inactivation of PS II finds new support and acceptance in recent work demonstrating that scavenging by tocopherol (a natural component of the chloroplasts) of $^1O_2$ generated by PS II *in vivo* protects PS II from PI (Trebst et al. 2002).

The idea that charge recombination via generation of the $P_{680}{}^{\cdot +}$/Pheo$^{\cdot -}$ radical pair either due to back electron flow in darkness from $Q_B{}^{\cdot -}$ or $Q_A{}^{\cdot -}$ semiquinone radicals or in light exposed membranes served as an incentive to study alternative pathways of energy loss via harmless back electron flow routes. Cytochrome b_{559}, a subunit of the PS II core com-

Figure 3e. A photograph of some of those involved in research concerning xanthophyll-cycle dependent photoprotection of Photosystem II (PS II), Budapest Hungary, 1998. *Left to right, first row:* Olle Björkman, Peter Horton, Harry Yamamoto and Agu Laisk. *Second row:* Govindjee (center with stretched arms), followed on the right by Adam Gilmore and A. V. Ruban. *Last row:* Roberto Bassi (second from the left), Joseph Hirschberg (last row, behind R. Bassi) and Krishna Niyogi (in striped shirt). Photo provided by Govindjee.

plex and mandatory for its stable formation (Pakrasi et al. 1989; Swiatek et al. 2002), had no measurable role in the PS II linear electron flow. However, experiments carried out by several groups indicated that back electron flow from the reduced side of PS II, possibly via cytochrome b_{559} to the oxidized PS II donor side may avoid charge recombination of the primary radical pair and thus may serve as a protective mechanism against PS II photoinactivation (Barber and De Las Rivas 1993; Poulson et al. 1995; Mor et al. 1997). One should mention at this point that photoprotection of PS II *in vivo* is provided also by lowering PS II excitation via thermal dissipation of the energy absorbed by the light harvesting antennae. This is achieved by transient changes in the xanthophyll content/composition of the thylakoid membranes, via the light activation of the xanthophyll cycle (for references, see Demmig-Adams et al. 1996; Horton et al. 1999; Niyogi 1999; Govindjee 2002; Govindjee and Seufferheld 2002). See Figure 3e for a photograph of some of the scientists working in the area of photoprotection of PS II.

While most of the PI studies converged on the understanding of the 'acceptor side photoinhibition' (Andersson et al. 1992), other mechanisms that may lead to loss of PS II charge separation and degradation of the D1 protein were considered as well. Removal of the manganese of assembled oxygen evolving complex in isolated thylakoids following Tris-washing allowed the possibility to study its rebinding and photoactivation of the water splitting process (Blubaugh and Cheniae 1990) as well as its effect on the light sensitivity of PS II (Eckert et al. 1991). Donor side inactivated PS II (Jegerschöld et al. 1990) was found to be significantly more sensitive to PI in low light, due to generation of long-lived $P_{680}{}^{+}$ and $Y_{161}{}^{+}$ radicals that could not be reduced in absence of donor side electron flow. While inactivation of the donor side does not occur *in vivo* under growth illumination conditions, lowering of the thylakoid lumen pH due to fast electron flow under strong illumination may result in release of Ca^{2+} that stabilizes the donor side resulting in down regulation of the oxygen evolving activity (Krieger and Weis 1993). This may contribute to the oxidative damage of PS II and thus D1 protein degradation. Several years passed before photoinhibition of donor-side inactivated PS II was addressed *in vivo*. The PS II donor side activity was impaired in absence of the enzyme processing the carboxyl terminal of the precursor D1 protein (pD1). This was the case in the low fluorescence *Scenedesmus* Lf1 mutant (Seibert et al. 1990) initially isolated by Norman Bishop (1962). Such mutants were considerably more sensitive to PI as compared to the wild-type cells when exposed to equal light intensities (Gong and Ohad 1995).

Already in the early stages of research of the fast turning over 32.5 kDa protein, Edelman and coworkers had shown that UVB light induces turnover of the protein (Greenberg et al. 1989). In combination with visible light UV has a synergistic effect on the protein degradation increasing the turnover of the protein (Jansen et al. 1996). Induction of PI by UV light was

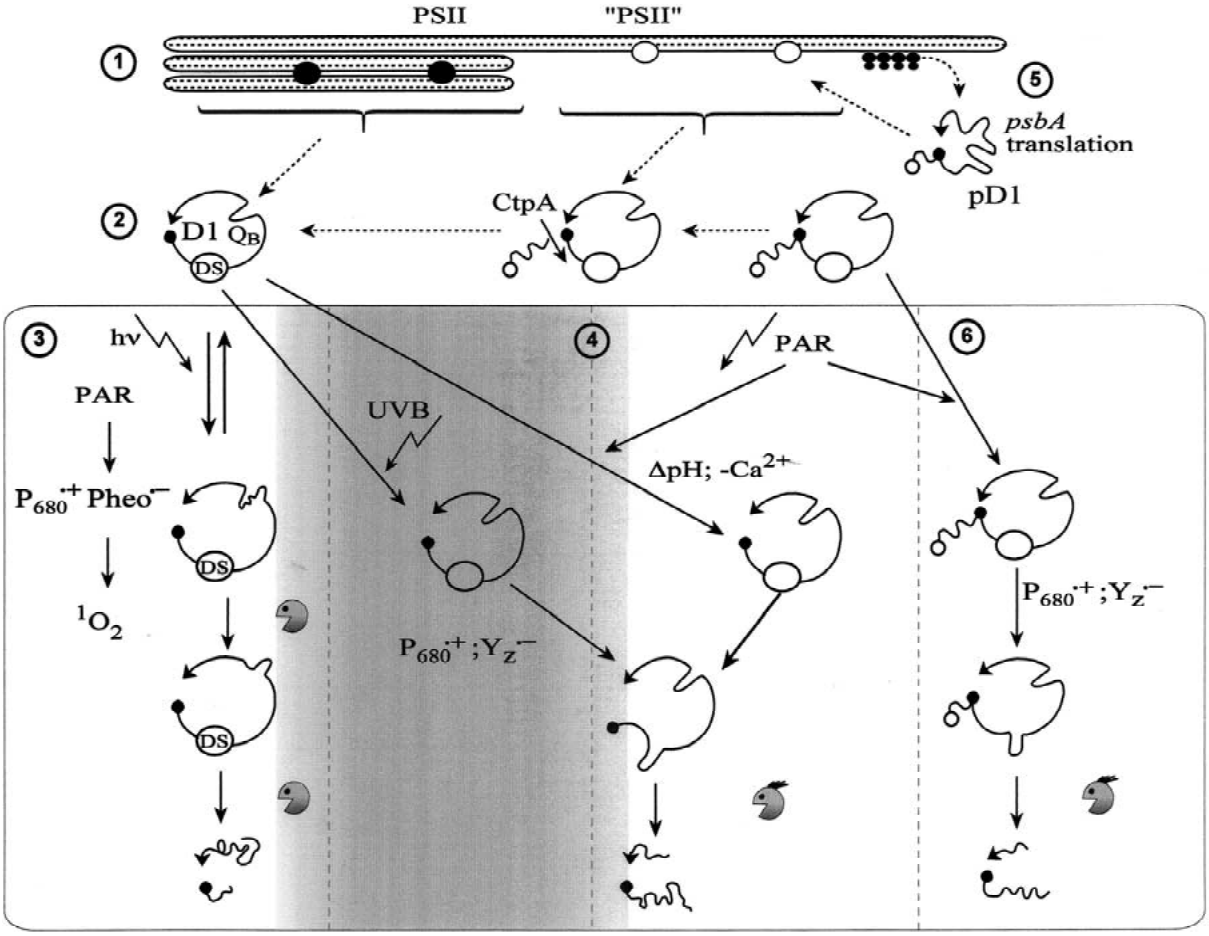

Figure 4. Pathways of visible and UVB (ultra violet B) light-induced damage to Photosystem II (PS II)-D1 protein in active PS II complex as well as during reassembly of PS II components with newly synthesized precursor D1 protein. (1) Thylakoid grana and stroma membranes; the black circles on the grana (appressed region) represent functional PS II; the open circles, located on the stroma membranes, represent residual PS II components ('PS II') in the process of assembly with newly synthesized precursor D1 protein (pD1); (2) D1 protein is represented by an open circle containing the Q_B binding site and the manganese cluster of the donor side (DS) complex; the arrow-head represents the N-terminal domain located on the stromal side of the membranes and the black circle at the protein end, the C-terminal domain located on the lumen side of the complex; (3) singlet oxygen ($^1O_2$) produced by charge recombination of $P680^{\cdot+}$ and $Pheo^{\cdot-}$ pair during exposure to photosynthetic active radiation (PAR, 400–700 nm) induces structural changes exposing the Q_B site to proteolysis in membranes undergoing acceptor-side photoinactivation; the first cleavage of the D1 protein in DE loop exposed on the stroma face of the membrane generates long N-terminal and short C-terminal fragments; (4) donor side inactivation is induced by UV light and/or by PAR; $P680^{\cdot+}$ and $Yz^{\cdot+}$ cation radicals generated in absence of electron donor activity result in oxidative damage and accessibility to cleavage of a lumen exposed D1 protein loop; a long C-terminal and short N- terminal fragment of the D1 protein are generated under these conditions; (5) ribosomes attached to the stroma membranes translate the *psbA* message generating pD1 that is inserted into the membrane; The precursor protein contains an extended C-terminal domain (marked by an open circle) that will be processed by the CtpA specific protease; pD1 can be assembled into PS II complexes prior to or after processing of the C-terminal domain; and (6) lin the process of assembly, 'PS II' containing pD1 lacks the donor side activity and is highly sensitive to photoinactivation by photosynthetically active radiation (PAR).

found to occur also at relatively low light intensities. Initially ascribed to sensitization of plastosemiquinone of PS II, it was found that the effect of UV might be related to the destruction of the manganese cluster as well (Vass et al. 1996, 2002). Unlike the case of the D1 protein degradation induced in acceptor side photoinactivated PS II, the initial cleavage of the protein in donor side photoinhibited PS II occurs at a protein site located on the lumen side of the membrane (Friso et al. 1994) possibly implying lumen localization of proteases. A schematic representation of the pathways of PS II photoinduced damage and D1 protein degradation is shown in Figure 4.

The D1 protein turnover cycle revealed

The results described above brought the study of PI from the realm of physiology to that of the PS II proteins. Having identified the lesion, the mechanistic questions could now be answered: How is the D1 damaged? Is there more than a single form of damage? How is the damaged D1 recognized for replacement? What is the mechanism of D1 degradation (single or multiple protease, peptidases)? Where does degradation take place? How does a newly synthesized D1 protein interact with a damaged reaction center II, RC II? Partial answers to these questions are represented schematically in Figure 5.

The answer to the question of what is the damage caused to the D1 protein was complicated by the findings of a number of research groups: the D1 protein can be found in the normal thylakoid membrane in a number of forms. As mentioned above, pD1 is cleaved on its carboxyl terminal to form mature D1 protein (Marder et al. 1984). This process is a prerequisite for the assembly of the manganese cluster and activation of oxygen evolution (Taylor et al. 1988; Ivleva et al. 2000). A transient population of palmitoylated D1 protein was identified in plants and algae (Mattoo and Edelman 1987; Adir and Ohad 1988). As mentioned above, the D1 protein is also transiently phosphorylated in most (Millner et al. 1986) but not all species (de Vitry et al. 1991; Pursiheimo et al. 1998;). Immunoblotting shows multiple D1 reactive bands. Thus, identification of a modified D1 species must be performed in a heterogeneous background. In cyanobacteria, multiple forms of the gene encoding the D1 protein, *psbA*, exist and their translation product and turnover is differentially light regulated (Schaefer and Golden 1989, see a photograph of Golden in the group shown in Figure 1a; Krupa et al. 1990; Sane et al. 2002).

However, the question arose whether any change in PS II could be identified prior to degradation that could be linked to PI and a number of laboratories followed the pathway of D1 turnover. Photoinhibition induces consecutive changes in the properties of the Q_B site gradually abolishing its protective effect against trypsin cleavage of the D-E exposed loops by biding of DCMU (Zer and Ohad 1995). In some experiments, populations of D1 protein covalently linked to the D2 protein or cytochrome b559 were indeed identified (Moskalenko et al. 1992; Ishikawa et al. 1999). An apparent covalent modification of the D1 protein, induced by high light treatment, was reported and proposed to be the first step towards turnover (Tal et al. 1999, see Figure 2d for a photograph of Tal). Noam Adir et al. (1990) as well as a number of other groups followed the pathway of D1 protein turnover. The D1 protein synthesis occurs on ribosomes bound to the stroma lamella membrane. Therefore, there must be a translocation mechanism allowing the newly synthesized protein to integrate into a preexisting PS II complex. Thus, one of the following *scenarios* could be envisaged: i) PI exposes the 'damaged' D1 protein to proteolysis leading to disassembly of PS II into subcomplexes; newly synthesized pD1 protein diffuses from the stroma lamella to the grana lamella and is assembled with the residual components of PS II into a functional complex (Figure 5), ii) Following photoinactivation the light harvesting complex (LHC II) dissociates from the photoinactivated PS II; cleavage of the D1 protein induces also the dissociation of the OEC proteins that are not degraded and may reassociate with a newly assembled PS II. The PS II complex further dissociates into subcomplexes that may diffuse laterally toward the stroma lamellae where either the pD1 or processed D1 protein may reassemble with PS II subunits; the reassembled complex diffuses within the membrane plane, and upon reaching the grana domain, reassociates with LHC II. Indeed this second possibility was shown to occur (Adir et al. 1990; van Wijk et al. 1994; Eisenberg-Domovich et al. 1995). Existence of free mobile populations of D1 protein could so far not be identified. These results also explained the existence of PS II β populations in the stroma lamella that increased under conditions of PI (Neal and Melis 1991). Studies of the translation and membrane insertion of the D1 protein in isolated developing chloroplasts disclosed that the translation process 'pauses' at specific points in the pD1 sequence elongation. Continuation of the translation activity requires supply of Chl and possibly cotranslation of the D2 protein as well (Muller and Eichacker 1999). These results as well as characterization of the PS II found in the stroma lamella (Kim et al. 1994) lead to the proposal that the amount of PS II RC found in the stroma lamella serves as a signal for measuring the amount of new D1 protein needed, thus providing a translation control mechanism allowing the translational machinery to 'feel' the light intensity.

The synthesis of PS II requires synthesis of Chl that is light dependent in higher plants. Synthesis of carotenoids is also required for the assembly of new reaction centers following photoinhibition (Trebst and Depka 1997).

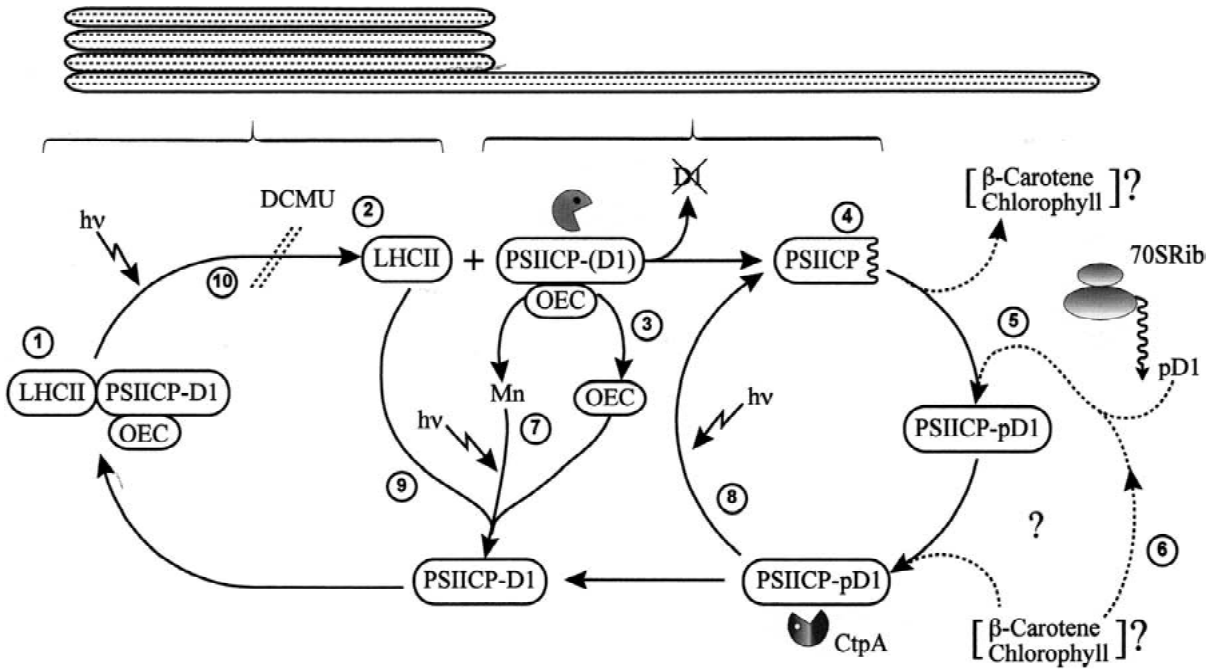

Figure 5. The repair cycle of irreversibly photoinactivated Photosystem II. (1) Grana domain region: PS IICP, Photosystem II core proteins; D1, the D1 protein; LHC II, the chlorophyll *a/b* light-harvesting complex; OEC, the proteins forming the oxygen evolving complex; (LHC II)(PS IICP-D1)(OEC), the assembled, functional complex. (2) The Photosystem II complex in the grana domains dissociates from the LHC II-antenna following photoinactivation and oxidative damage to the PS II core complex. (3). The D1 protein of the modified PS II core (PS IICP-(D1)) is exposed to proteolytic degradation, resulting in the dissociation of the oxygen evolving complex (OEC) proteins and their release into the thylakoid lumen. (4) The residual PS II core proteins (PS IICP) migrate laterally to non-appressed domains of the membrane and chlorophyll *a* and β-carotene may be released from the reaction center core residual complex during the degradation of the D1 protein. (5) The residual components of the PS II core may reassemble with the precursor of the D1 protein, pD1, synthesized by membrane bound 70s ribosomes. (6) Chlorophyll *a* and β-carotene rebind following reassembly of PS II that still lacks the donor side activity prior to the pD1 processing (PS IICP-pD1). (7). PS II activity is reestablished following processing of the pD1 protein, reassociation of the OEC complex, binding and formation of the manganese cluster and its photoactivation. (8) The donor side inactive Photosystem II (PS IICP-pD1) is highly sensitive to light and the protein rapidly degraded if pD1 is not processed. (9) The reassembled active PS II complex may migrate laterally to the appressed domains and may reassociate with the LHC II antennae. (10) Binding of DCMU (3-(3,4, dichlorophenyl) 1,1-dimethylurea), triazine-derived herbicides or absence of plastoquinone, retard the D1 protein degradation of the photoinactivated PS II. Scheme drawn for this review, modified from Figure 3 in Keren and Ohad (1998).

Since its early stage, one of the confusing aspects of PI and D1 protein turnover research was the apparent lack of proteolytic fragments that should have appeared during PI. Indeed, experiments performed with very high levels of radioactively labeled D1 protein, which was completely degraded by the end of the PI treatment, showed no fragments at all. This indicated that the degradation of the fragments generated by the initial D1 protein cleavage must occur at a very high rate, possibly by a battery of proteases. The first sign of fragments of the D1 protein seen *in vivo* was reported by Bruce Greenberg (Greenberg et al. 1987, see Figure 1e for a photograph of Greenberg), using a combination of labeling and protein synthesis inhibitors. However, resolving the degradation problem led to increased efforts to develop *in vitro* PI systems that resulted in the detection of multiple forms of D1 protein altered and thus prone to exposure of the cleavage site to proteolysis even though such PS II populations did not lose activity (Spetea et al. 1999).

The events leading to the degradation of the D1 protein and the PS II 'repair cycle' as described above are schematically summarized in Figures 4 and 5.

PI as understood from *in vivo* versus *in vitro* studies

As with most other photosynthetic phenomenon, there was a strong motivation to investigate PI in a more limited and controlled background, which could have potentially led to a more detailed description of the

entire process. The research pathway of system fragmentation and isolation resulted in a detailed understanding of the separate functional details of each of the steps of photosynthesis, and their integration in a general scheme allowing the reconstruction of this complicated energy transducing system.

In the case of the study of PI, moving from *in vivo* systems to *in vitro* systems was deceptively simple. The methodologies for different levels of system isolation existed: whole chloroplasts, thylakoid membranes, holo-PS II (RC II with a full complement of Chl *a*-antennae), O_2 evolving RC II or RC II cores. These could be isolated, and illuminated with intense light. And as expected, PI was induced and the D1 protein was lost from its normal position on SDS-PAGE and immunoblots. In some cases, secondary effects occurred under the conditions used, such as artifacts due to the formation of insoluble protein aggregates. In one case, where the general cross-linking reagent glutaraldehyde was used to form intramolecular cross-links in all of the thylakoid membrane proteins, high light treatment appeared to induce the loss of the D1 protein, in a fashion similar to photoinhibitory treatment of whole cells. However, in this case, as well as in other *in vitro* experiments, the D1 protein actually formed insoluble aggregates with the other PS II proteins, and thus 'disappeared' from the SDS-PAGE or immunoblot analysis (Adir and Ohad 1988). These results led to the question whether *in vitro* high light exposure indeed reproduced the effects of similar treatment *in vivo*. Despite the above-mentioned difficulties, *in vitro* PI experiments provided a number of observations, which correlated well with the results, obtained *in vivo*. These observations have been previously reviewed in detail (Barber and Andersson 1992; Prasil et al. 1992; Aro et al. 1993; Keren and Ohad 1998). Briefly, the following conclusions could be obtained:

i) PS II can be damaged by high light treatment *in vitro* by over reduction of the plastoquinone pool that leads to acceptor side induced PI. Lack of plastoquinone leads to loss of Q_B function, followed by over-reduction of Q_A (Styring et al. 1990); this can either damage PS II directly, or by virtue of its loss, induce formation of damaging species following increased charge recombination rates.

ii) Loss of electron transfer from water via the oxygen evolving complex (OEC) leads to donor side induced PI. In this case damage is probably caused by the persistence of highly oxidizing species such as $P_{680}{}^{+}$ or $Tyr_z{}^{+}$

iii) Inactivation of PS II *in vivo* precedes the loss of D1 protein. High light exposure at low temperature inhibits the protein degradation, but not its damage. Following transfer of the system to higher temperature in darkness results in the loss of the D1 protein (Aro et al. 1993).

iv) In both forms of PI, the D1 protein is fragmented. These fragments are generated by proteases, which recognize the modified RC II-D1; the first cleavage has been considered also to be possibly due to a photochemical effect. However, thylakoid membrane proteases have been recently characterized (Lindahl et al. 1995; Adam 2000; Lensch et al. 2001), and a putative D1 protein specific protease has been identified (Haussuhl et al. 2001).

v) In addition to the loss of the D1 protein, PI-induces degradation, although at lower rates, of other PS II proteins including the D2 protein, cytochrome b_{559}, CP43, CP47 (Schuster et al. 1988; Yamamoto and Akasaka 1995; Zer and Ohad 1995; Jansen et al. 1999; Ortega et al. 1999). These results are important, since they show the 'evolutionary motivation' for the D1 turnover process – when damage exceeds turnover, the other RC II proteins are damaged, resulting in the need for *de novo* synthesis of the entire RC; this may create a problem of large protein aggregates, which cannot easily be degraded and thereby may reduce the viability of the photosynthetic apparatus.

The present 'state of the art' and perspectives for the study of PI

The investigation of the irreversible photoinactivation of PS II, degradation of its damaged core subunits and reassembly of the residual complex components with newly synthesized ones, the so called repair cycle proposed in 1984 (Ohad et al. 1984), that was pursued during the period of almost two decades since middle of 1980 has established the major features of these phenomena.

The general interest aroused by the initial findings of the light induced turnover of the D1 protein and its relation with the loss of PS II activity has attracted many researchers and many laboratories became involved in the study of various aspects of PI. The Harden Conference dedicated to PI in 1994 resulted in the publication of 'Photoinhibition of Photosynthesis

from Molecular Mechanisms to the Field' (Baker and Bowyer 1994), see Figure 3c for a photograph of the participants) and represented a culminating point in this field of research. One of the major sources of controversy in the study of PI has always been the question of its physiological relevance. As already noted above, PI occurs in different plants at different light intensities, since each organism is typically pre-adapted to a certain light-regime. Thus, desert plants that are exposed to very high light intensities during much of the day rarely die of PI. However, it is quite clear that while PI was recognized as a physiological phenomenon that exists primarily in the laboratory, its understanding required a detailed description of photosynthesis in molecular terms which must take into account that PS II is a highly dynamic heterogeneous complex. The lack of knowledge of its precise structure has limited the progress of the PI research that reached a plateau and even certain stagnation in the later half of the 1990s. Interest in the field became limited and many of the leading laboratories shifted their interest towards more physiological or structural studies. One of us (I.O.) remembers vivid discussions among many colleagues on the need to continue and struggle to investigate PI and there were those who even doubted that its understanding could contribute significantly to the study of PS II under the conditions prevailing at that period. On one such occasion Achim Trebst argued that 'the field is tired of photoinhibition'and there are certainly more interesting topics pertaining to PS II to investigate and at that point he was certainly right. The paradox is that recently Trebst himself has revived the field by providing new insight into the protection of PS II by tocopherol against oxidative damage induced by singlet oxygen (Trebst et al. 2002). This work was made possible by the recent progress in the resolution of PS II structure as well as carotenoid metabolism (Hirschberg 2001). The realization of the complexity of PS II function as revealed by PI research has had an impact on many other fields of photosynthetic research: charge separation, energy and electron transfer, oxygen evolution, regulation of forward and backward electron flow and mode of action of PS II herbicides, to mention only a few.

It is evident that a complete understanding of PI and the repair process requires a detailed analysis of each step while using advanced technology that is now available. Identification of the primary oxidative events leading to the inactivation of the acceptor or donor side need not be ascribed solely to modification of the D1 protein. The heterodimer formed by the D1/D2 proteins and Cytochrome b_{559} and 10 kDa protein acts as an entity capable of charge separation and thus one can not exclude the possibility that oxidation of the D2 protein may trigger the conformational change that exposes the cleavage site of the D1 protein to the initial cleavage step. Furthermore, the possibility should be considered that the oxidative damage might primarily affect other PS II proteins. These could include the low molecular weight (LMW) single transmembrane subunits of PS II, PsbJ, PsbL, PsbI, as well as other small polypeptides. The structure of cyanobacterial PS II is presently resolved at 3.8 Å (Zouni et al. 2001) and the resolution of the chloroplast PS II has progressed reaching the 7–8 Å mark (Boekema et al. 1995; Nield et al. 2000). However, the identity and exact location of the LMWs in the PS II structure have not yet been precisely resolved. The possible light induced alteration of these proteins may affect the structure of the heterodimer and should not be ignored. Suggestions for such possibilities may be found in the work of Nakajima et al. (1996) and Cornelia Spetea et al. (1999) who reported that herbicide binding to the Q_B site exposes a fraction of the membrane D1 protein of dark adapted membranes to the primary cleavage in darkness. Short exposure to weak light, far from inducing photoinactivation of PS II, generates different forms of cleavable D1 protein, some reversible in darkness. Recent studies have demonstrated that inactivation of the *psbJ* in tobacco resulting in the loss of a single transmembrane protein of 3.5 kDa, associated with PS II, leads to the formation of PS II capable of charge separation and reduction of Q_A, but incapable of reducing plastoquinone, thus mimicking the acceptor side-photoinhibited PS II (Regel et al. 2001). Rapid isolation of PS II containing 32 identified protein subunits, by the use of poly-histidine tagged PS II-CP43 and nickel columns, permits the isolation of each of the PS II proteins (Kashino et al. 2002) and proteomics techniques in combination with mass-spectrometry may allow us to identify even minor modifications of each of the proteins at various stages of the PI process. Using a similar approach, it will be possible to analyze the composition of subcomplexes formed during the dissociation of the PS II complex undergoing the first steps of PI, as well as the pattern of re-assembly during the repair process. Results to be obtained by this approach are expected to generate surprises that may change the basic dogmas now ruling the present understanding we have of PI and repair processes.

For many years, the light-induced turnover of the D1 protein was regarded from the point of view of light induced damage to PS II. We dare to take a more positive outlook on this process: the turnover of the D1 protein may be just another mechanism of PS II protection against light induced oxidative stress. As in all forms of oxidative stress studied to date, they depend on environmental factors which if not changed to highly detrimental conditions, can be coped with relatively well at all light intensities at which plants are adapted in their normal distribution on our planet.

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