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Review

Photoinhibition of Photosystem II. Inactivation, protein damage and turnover

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Contents

Summary

Even though light is the source of energy for photosynthesis, it can also be harmful to plants. Light-induced damage is targetted mainly to Photosystem II and leads to inactivation of electron transport and subsequent oxidative damage of the reaction centre, in particular to the D1 protein. Inactivation and protein damage can be induced by two different mechanisms, either from the acceptor side or from donor side of

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P680. The damaged D1 protein is triggered for degradation and digested by at least one serine-type proteinase that is tightly associated with the Photosystem II complex itself. The damaged Photosystem II complex dissociates from the light-harvesting antenna and migrates from appressed to non-appressed thylakoid regions where a new D1 protein is co-translationally inserted into the partially disassembled Photosystem II complex. D1 protein phosphorylation probably allows for coordinated biodegradation and biosynthesis of the D1 protein. After religation of cofactors and assembly of subunits, the repaired Photosystem II complex can again be found in the appressed membrane regions. Various protective mechanisms and an efficient repair cycle of Photosystem II allow plants to survive light stress.

I. Introduction

Light is the driving force of photosynthesis and therefore an absolute prerequisite for the autotrophic growth of photosynthetic organisms. However, light is an elusive substrate that is not easily managed at the molecular level, and that can also be harmful to the photosynthetic apparatus. Photosynthetic organisms therefore have to manage the task of maintaining sufficient excitation power under limiting light conditions as well as avoiding photodamages at high light.

There is now a general consensus that the main target for light stress in organisms possessing oxygenic photosynthesis is Photosystem II (PS II) [1], leading to impairment of electron transport and irreversible damage to reaction centre subunits, in particular the so-called D1 protein [2]. Plants survive this damage through a costly and complicated repair process that involves biodegradation and de novo protein synthesis. The half-time of D1 protein turnover can be as short as 30 min [3,4]. This can be directly illustrated by pulse-labelling of mature leaves showing that the D1 protein is one of the main proteins synthesized in the light.

Light stress to PS II in vivo only becomes a problem for the plant in terms of photosynthetic capacity when the rate of photodamage exceeds the capacity of the repair process. This stress situation is known as photoinhibition and can lead to a reduction in plant growth [5,6]. The susceptibility of plants to photoinhibition at a given light intensity varies widely with the genetic adaptation, physiological state and life history of the plants. Moreover, photoinhibition as a physiological phenomenon is also greatly dependent on other environmental conditions besides light. In particular, photoinhibition is aggravated if high light is combined with other stress factors such as low or high temperature, drought or CO_2 deficiency and can then occur at moderate light intensities.

In order to avoid or minimize photoinhibition, plants have evolved several strategies. The main one is the repair of PS II via replacement of damaged reaction centre proteins [7,8]. Other protective mechanisms are more prophylactic and involve dynamic reversible changes in antenna size of PS II and can be both of short-and long-term nature [9]. Moreover, thermal dissipation of excess energy is also thought to serve a major protective role and may occur either in the antenna bed or in the reaction centre [10–13].

The intriguing nature of the photoinhibition problem has attracted much physiological research over the years. However, today we are experiencing a new development, with many scientists approaching the photoinhibition problem at the molecular level in order to understand the detailed mechanism of the various inactivation and damaging steps. Today, photoinhibition is one of the major topics of photosynthesis research. The main reason for this development is that the understanding of the structure and function of the PS II reaction centre is now very advanced, mainly because of the functional [14] and structural analogy [15, 16] with the reaction centre of the photosynthetic purple bacteria, whose three-dimensional structure has been determined [17]. Thus, in comparison with several other areas of biological stress research, very specific molecular questions can be asked and be subjected to detailed experimental analysis.

This review will focus on recent achievements based mainly upon in vitro studies regarding the molecular knowledge of photoinhibition of PS II. The process will be described as a series of events that include photoinactivation of electron transport, irreversible photodamage of reaction centre components and biodegradation, which are followed by a repair process that involves synthesis of new D1 protein.

II. The Photosystem II complex – a structural and functional background

A detailed discussion on the mechanisms for photoinhibition requires a short background description of our knowledge about PS II. The characterization of this essential biological complex has been most successful. Apart from the detailed model of the PS II reaction centre, during the last 10–15 years at least 25 polypeptides have been assigned to the complex [18] (Fig. 1). The PS II complexes are embedded in the thylakoid membrane and are located mainly in its appressed regions while only a small fraction is located in the stroma exposed thylakoid regions together with PS I and the ATP synthase [9].

The initial event in PS II is the capture of light by chlorophyll binding proteins. The main portion of the light-harvesting antenna is made up of several chlorophyll *a*/*b*-proteins, mainly LHC II, but there are also two major chlorophyll *a* proteins (CP47 and CP43) [19] which are more tightly associated with the reaction

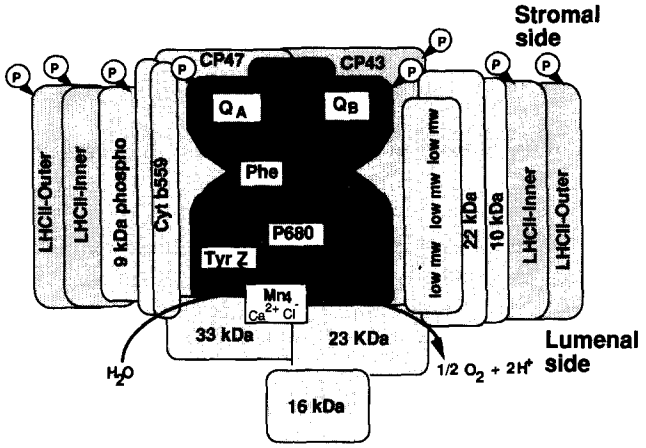

Fig. 1. A schematic presentation of PS II in higher plants, including major polypeptides and cofactors involved in PS II photochemistry. The central part of the figure shows the D1/D2 protein heterodimer with bound redox components. The arrows indicate the electron transfer pathway from water to the secondary quinone acceptor, Q_B. The manganese cluster participating in oxidation of water is placed on the D1 protein. Polypeptides and cofactors are marked with their molecular mass or abbreviations. LHC: Light-harvesting complex, Low mw: a number of polypeptides with the molecular weight ranging from 7 to 3 kDa, so far with virtually unknown function. CP: Chlorophyll protein.

centre (Fig. 1). The light energy captured by the antenna is transferred to the primary electron donor $P680^+$, most likely a chlorophyll a dimer [20]. The excited $P680^+$ transfers one electron to a pheophytin, the primary electron acceptor. This primary charge separation is stabilized by fast electron transfer to a plastoquinone (Q_A) resulting in the radical pair $P680^+$-Q_A^- being formed. In a slower reaction, the electron is transferred to the secondary plastoquinone acceptor (Q_B). After a new light-induced charge separation reaction Q_B^- receives a second electron followed by protonation forming plastoquinol (PQH_2). The reduced plastoquinone is displaced from the reaction centre to intermix with the plastoquinone pool of the thylakoid membrane which is involved in further redox interactions with the cytochrome *b/f* complex. The $P680^+$ is an exceptionally strong oxidant (+1.1 V) and is thus able to extract electrons from water [20,21]. This occurs via a redox-active tyrosyl residue (Tyr_z) and a cluster of 4 manganese atoms. During the oxidation of water the manganese cluster cycles between five different redox states (S_0–S_4) to release one molecule of oxygen.

Despite the complicated array of electron transfer

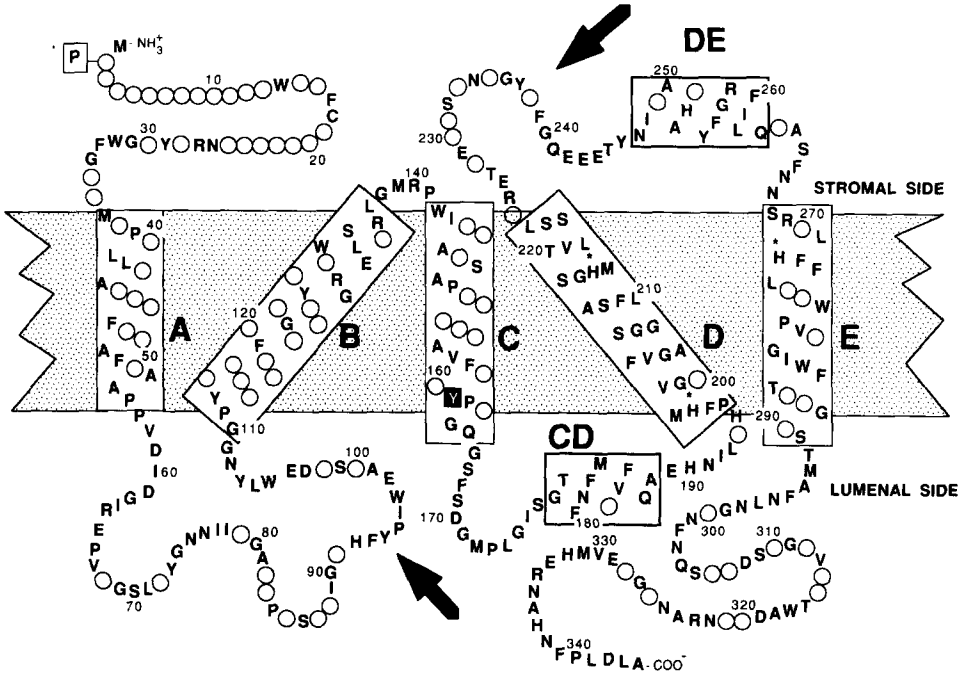

Fig. 2. Predicted folding pattern for the D1 protein from spinach. The protein sequence from spinach was compared to 37 other sequences for the D1 protein from other species [31,32]. The arrows indicate possible proteolytic cleavage sites following photodamage. The residues conserved in all published D1 sequences are shown here by their one letter symbols while the circles represent non-conserved residues. The sequence numbering corresponds to the D1-sequence from spinach. Tyr 161 which has been identified as the primary electron donator to P680, Tyr_Z, is marked with a white letter on dark background. Histidine residues that are supposed to participate in binding of the acceptor-side iron and the Mg ions in the primary donor are marked with asterisks. The B and D helices are drawn tilted, although in the structure only helix D is expected to be tilted with respect to the membrane plane. The phosphorylation site which is at a threonine residue adjacent to the *N*-terminal is also shown. Adopted from Bengt Svensson.

reactions and the very large number of subunits in PS II, only two subunits, the Dl and D2 proteins, appear to ligate the redox components required for the water-plastoquinone oxido-reduction reaction. For the acceptor side, this assertion is based primarily on the concept of functional [14] and structural [15,16] analogy between the L and M subunits of the reaction centre in photosynthetic purple bacteria and the D1 and D2 proteins of PS II. The isolation of a protein complex from plant thylakoids capable of primary charge separation and comprised of the D1 and D2 proteins in addition to cytochrome *b*-559 and the *psbI* gene product added important biochemical support for this idea [22,23].

In contrast to the situation on the acceptor side, the donor side of PS II exhibits no functional similarity with the bacterial reaction centre. However, recent experiments have shown that the components of the PS II donor side are also associated with the D1/D2 protein heterodimer. Tyr_z has been shown by site-directed mutagenesis to be tyrosine 161 of the D1 protein [24,25]. Moreover, several experimental indications [26–30] and theoretical predictions [31,32] raise the possibility that the reaction centre subunits, particularly the D1 protein, harbour the 4 manganese atoms of the water splitting-system.

In light of these significant developments we can now view the PS II reaction centre as a heterodimer comprised of the D1 and D2 proteins. Each of these two proteins possesses five transmembrane helices with the N- and C-terminal portions at the outer and inner sides of the thylakoid membrane, respectively (Fig. 2). It has even been possible to assign functionally distinct amino acid residues within the PS II reaction centre [15,16,33]. The most crucial ones are the three histidines in each of L and M proteins, which correspond to His-198 (D1 and D2) His-215 (D1 and D2) His-272 (D1) and His-269 (D2). The last group of four histidines are thought to coordinate the acceptor-side iron (Fig. 1), while P680 binds to His-198 in the D1 and D2 proteins. The primary acceptor pheophytin is assumed to bind to the B helix of the D1 protein close to the stromal side of the membrane involving Glu-130. The primary quinone Q_A is considered to bind in the stromal loop connecting the D-E helices of the D2 protein, while the secondary quinone Q_B is thought to bind in the corresponding loop of the D1 protein.

The concept of the PS II reaction centre being comprised of a D1/D2 protein heterodimer is one of the most important developments in photosynthesis research during recent years. Of interest, particularly with respect to the photoinhibition problem, is that the D1 protein prior to its assignment as a reaction centre subunit was termed the '32 kDa-Q_B binding'-, the 'herbicide binding'-, or the 'rapidly-turning over protein' [34,35]. It was given a role at the acceptor side of PS II as a carrier of the secondary plastoquinone acceptor Q_B, but as discussed above we now know that this protein has a much more central role in PS II.

III. Photoinhibition of Photosystem II – an overview of events

The vulnerability of PS II to light stress manifests itself as photoinactivation of electron transport and irreversible photodamage to the reaction centre proteins. This vulnerability is thought to be a reflection of the complicated chemistry underlying the light-mediated water-plastoquinone oxido-reduction reaction. Several problems connected to this process can be pinpointed. Light as a substrate is not easily managed so that light-harvesting and primary photochemistry go on even when other metabolic reactions are limiting. Oxygen, as one of the products, can moreover form highly toxic species in the presence of excited pigments or when redox reactions are occurring. Still another problem is that very high potential oxidizing intermediates are formed, possessing potentials as high as over 1.1 V, which in turn can give rise to oxidative damage to neighbouring molecules.

This type of photoinhibition process, leading to destruction of the PS II reaction centre, should not be confused with reversible down regulation of PS II which is due to quenching of excitation energy in the reaction centre [13] and/or the antenna bed [10,11].

The '32kDa-Q_B binding protein', now known to be the D1 protein, of the PS II reaction centre was suggested to be involved in the photoinhibition process at an early stage [36,37]. This was based mainly upon the connection between an increased turnover of the Q_B-protein at high light intensities and on the observation that recovery from photoinhibition required de novo synthesis of the Q_B protein. The protective effect on photoinhibition by certain herbicides, such as DCMU and atrazine, which bind to the 32 kDa Q_B^- protein and displace the plastoquinone, was also taken into account [38,39]. Thus, damage to the 32 kDa protein was thought to be the primary cause for photoinhibition. At this stage is was suggested that quinone anions reacting with molecular oxygen would produce oxygen radicals, which in turn would lead to protein damage, thereby inhibiting electron transport [36].

Since these important pioneering studies on the molecular mechanisms behind photoinhibition, the concepts have changed and have, in fact, become inverted. There is currently a general consensus that the light-induced impairment of electron transfer is not a consequence but rather the cause of protein damage and turnover (see below). Most importantly for the more recent developments in photoinhibiton research is the model of a D1/D2 protein heterodimer as the PS II reaction centre. (Fig. 1). It is now apparent that

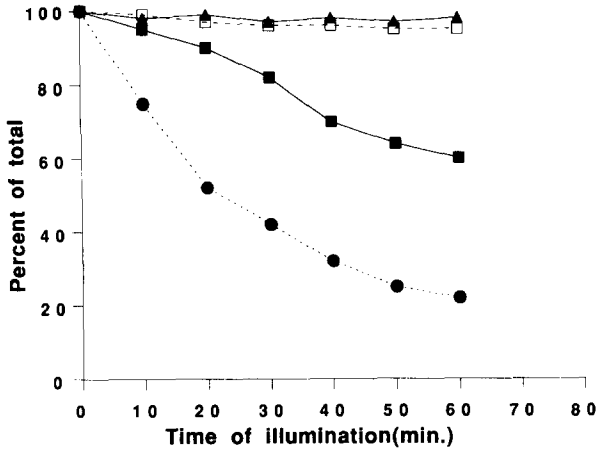

Fig. 3. The effect of illumination at various conditions on isolated thylakoid membranes. (▲) D1 protein degradation under anaerobic conditions, (□) D1 protein degradation at 2°C, (■) D1 protein degradation at 20°C, aerobic conditions, (●) oxygen evolution at 20°C, aerobic conditions.

the rapidly turning-over 32 kDa protein is not only associated with acceptor-side electron transport but actually essential for all electron transfer reactions in PS II including water oxidation. The heterodimer model also identifies a new level of complexity of the photoinhibition problem. How is the functional integrity of the PS II complex affected, in terms of protein assembly and ligation of cofactors, when the D1 reaction centre subunit is specifically undergoing rapid turnover?

In an important paper by Arntz and Trebst [40], it was demonstrated that photoinactivation of PS II electron transport under anaerobic conditions did not result in D1 protein degradation. Experiments using strong illumination of isolated thylakoid membranes in the presence of oxygen showed that the rate of impairment of electron transport clearly exceeded that of D1 protein degradation [28,41] (Fig. 3). Moreover, if the photoinhibitory illumination was applied to isolated thylakoid membranes at low temperature there was no D1 protein degradation despite a pronounced impairment of the electron transport [42] (Fig. 3). However, if thylakoids inhibited at low temperature were transferred to room temperature in absolute darkness, the D1 protein degradation started without any further loss of electron transport activity. There was also a direct correlation between the amount of D1 protein degraded in the dark and the extent of inactivation during the preceding period of light stress in the cold. This experiment, apart from corroborating the initial role of the electron transport inactivation, shows that the subsequent D1 protein degradation reaction per se does not require light and gives support for an enzymatic reaction rather than a photocleavage. Moreover, it implies some kind of 'molecular memory' or 'triggering' that couples light-induced inhibition of electron

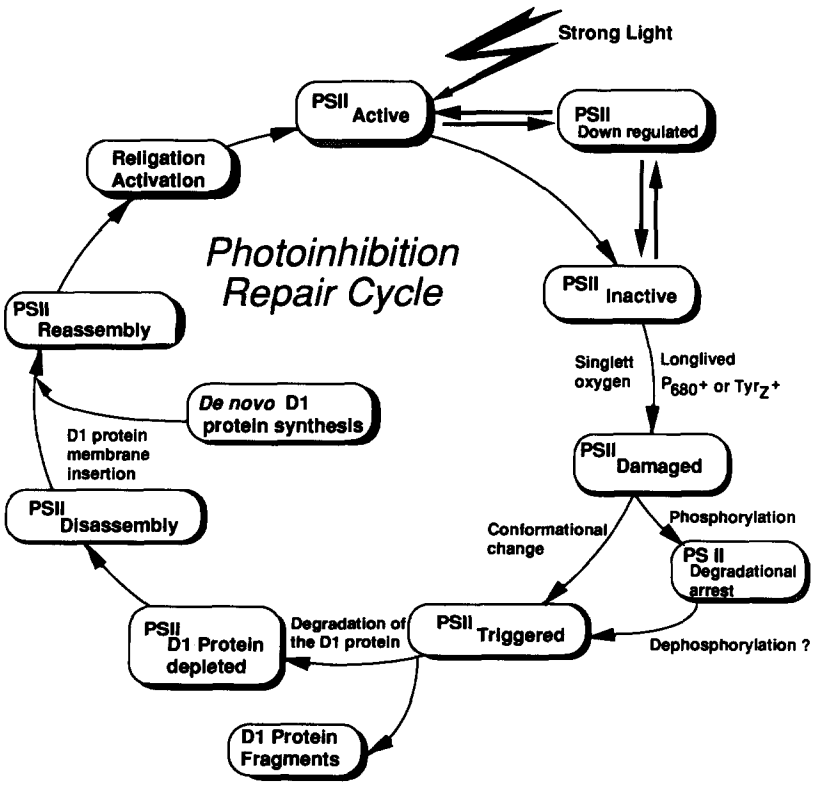

Fig. 4. This hypothetical scheme shows the different phases involved in photoinhibition and the subsequent repair mechanism.

transport with proteolytic removal of irreversibly damaged D1 protein.

As will be discussed in the next sections, there is now strong evidence that the photoinhibition of PS II can be viewed as a sequential series of events (Fig. 4). These include light-induced impairment of electron transport (Section IV), irreversible damage to the PS II reaction centre (Section IV), triggering of the D1 protein for degradation (Section V), proteolytic cleavages (Section V), secondary changes to the PS II complex (Sections VI and VII) and finally biosynthetic reactions to reestablish functional water-plastoquinone oxido-reduction (Section VIII) [2,18,43]. More and more evidence is also accumulating to suggest that there are at least two mechanisms for photoinactivation (Section IV), one induced from the acceptor side of PS II and another from the donor side [2,18,43,44]

IV. Mechanisms for photoinactivation of Photosystem II electron transport and protein damage

IV-A. Acceptor-side-induced photoinactivation

When photosynthetic tissue, cells, isolated thylakoid membranes or even subthylakoid preparations are subjected to light intensities above saturation level several characteristics of PS II are changed. Apart from a reduction in the quantum yield of oxygen evolution there are changes in the fluorescence parameters, changed properties of several EPR signals, altered

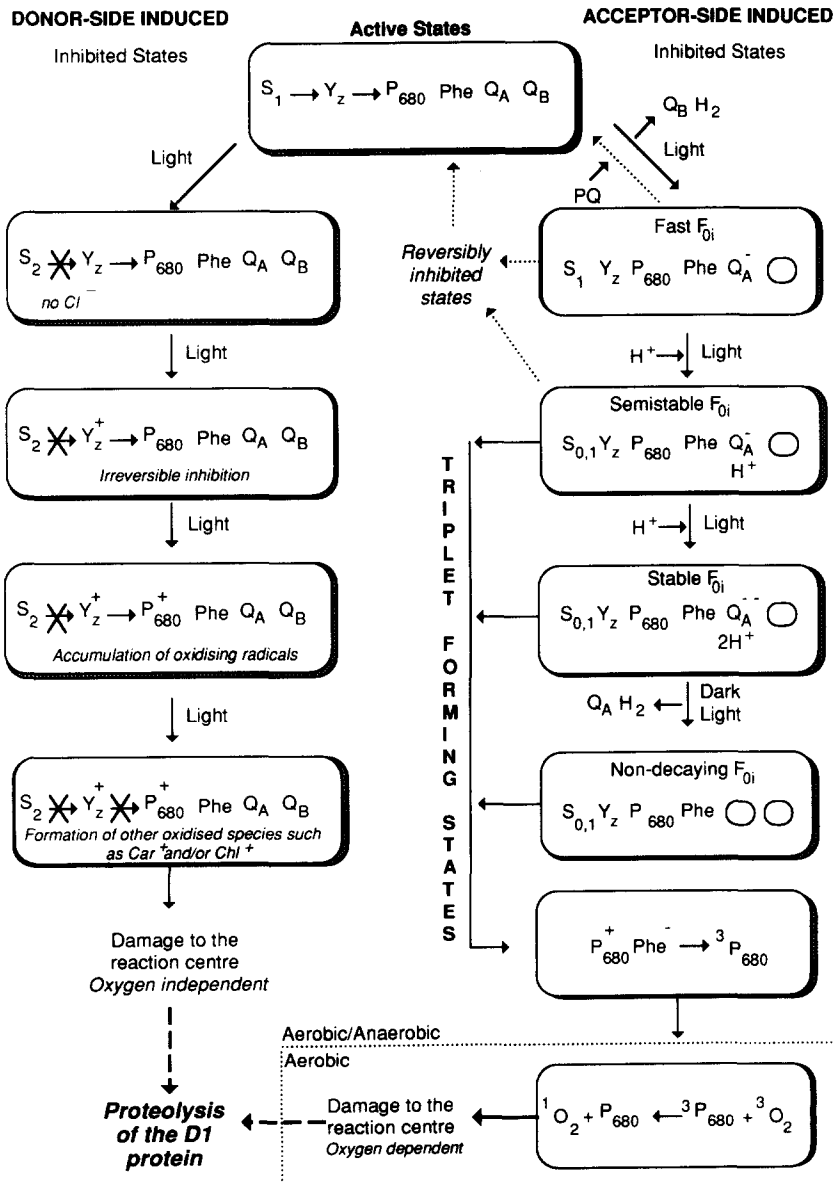

Fig. 5. A hypothetical scheme showing various intermediates under acceptor-side and donor-side inactivation.

thermoluminescence characteristics, transformation of cytochrome *b*-559 from high to low potential form and finally protein degradation [2,18]. Despite a wealth of experimental data, it has not been easy to resolve experimentally in detail the primary inactivation events from the secondary. However, the majority of the present results suggest that photoinhibition can be initiated at the acceptor side of PS II at the level of Q_A and/or Q_B.

One approach that has been fruitful is to follow the photoinactivation process in vitro under anaerobic conditions. The advantages of this anaerobic approach are that there is normally no degradation of the D1 protein in the absence of oxygen (Fig. 3) [40,45–47] and that the inactivation is reversible in its early stages without de novo protein synthesis [45,48]. It is therefore possible to trap and characterize intermediate and reversible stages in the photoinactivation process. In a recent study it has been possible to give a quite detailed model for the course of events during acceptor-side-induced inhibition and protein damage using this anaerobic approach combined with a variety of analyses including fluorescence and EPR spectroscopy [49–52]. We will below use this model as a basis for further discussion on the mechanism of acceptor-side-induced photoinactivation.

As illustrated in Fig. 5 the photoinactivation process can be resolved into four phases which are based upon the characteristic decay kinetics of the elevated Fo fluorescence after anaerobic photoinactivation. The four photoinactivation intermediates have been designated fast ($t_{1/2} = 30$ s), semi-stable ($t_{1/2} = 2$ min), stable ($t_{1/2} = 30$ min), and non-decaying [49] according to their F_o fluorescence decay times. Moreover, kinetic investigations of the F_o fluorescence after different periods of strong illumination revealed that the various inhibition intermediates were formed in a sequential manner in the order given above. Previous studies [48,53] had observed the stable and non-decaying intermediates but could not resolve the fast and semi-stable ones. Each of the four photoinactivation intermediates revealed distinct variations in their acceptor-side properties (see below), suggesting that a series of consecutive reactions lead to irreversible impairment of the PS II electron transport and, if oxygen is present, to protein damage and degradation (Fig. 5).

Initially, the strong illumination leads to an over-reduction of the plastoquinone pool in the PS II membranes, which most likely leaves the Q_B-site non-operational due to a lack of reducible plastoquinone molecules [49]. This was deduced from the loss of the rapid and medium phases of fluorescence decay after a single turn-over flash according to Crofts et al. [54]. In turn, this most probably leads to a stabilization of the singly-reduced primary quinone acceptor (Q_A^-), which increases its half-life time to 30 s as compared to a few hundred ms for normally turning over Q_A^-. Further illumination transforms this 'fast' Fo inhibition intermediate into the semistable intermediate in a reaction which is facilitated at low pH. It is likely that the relatively long life-time of the reduced Q_A^- species during the fast intermediate phase allows protonation of Q_A^- to occur thereby providing further stabilization and transformation into the semi-stable state. A striking feature of this semi-stable intermediate is a dark stable Q_A^- Fe^{2+} EPR signal. This EPR signal is only obtained when Q_A is single reduced while oxidized or double reduced Q_A are EPR silent [55]. In non-photoinhibited, dark incubated thylakoids the Q_A^--Fe^{2+} EPR signal is not present at room temperature since Q_A^- is rapidly reoxidized, but it can be visualised by illumination at 77K. Thus, the detection of the Q_A^--Fe^{2+} signal in the dark of PS II preparations subjected to high light strongly indicates that stabilized Q_A^--species are intermediates in the photoinactivation process.

The further transition from the semi-stable to the stable intermediate (Fig. 5), which is also promoted at low pH, occurs concomittantly with the loss of the dark stable Q_A^--Fe^{2+} EPR signal. This result strongly suggests that Q_A has become either double reduced and/or has left its binding site in the reaction centre. The reversibility of this stable photoactivation intermediate [45,48,49] favours the former situation, where a double reduced and protonated Q_A remains bound to the D2 protein.

The final photoinactivation intermediate, which is the first irreversible one under anaerobic conditions, is characterized by non-decaying Fo fluorescence and the absence of inducible Q_A^--Fe^{2+} EPR signal. This non-decaying state is suggested to contain reaction centres with an empty Q_A-site [49,56] due to the release of double-reduced plastoquinone in analogy with what has been proposed to occur during chemical double reduction of Q_A [57]. Quite recently, direct support for the possibility of Q_A release during light stress of PS II has been obtained by HPLC analyzes of the plastoquinone content in PS II core particles which were isolated from membranes that had been subjected to photoinhibitory treatment under anaerobic conditions [52]. These analyses revealed that the amount of Q_A lost from the photoinactivated PS II complexes correlates with the proportion of non-decaying centres.

The recovery of photoinactivation from the three first intermediates under anaerobic conditions can be almost complete [49]. Notably, DCMU prevents reversibility for all three states which suggests that the recovery process involves re-establishment of Q_A to Q_B electron transfer after re-oxidation of the PQ pool and re-occupation of the Q_B site. For the semi-stable and stable intermediates, the recovery also seems to require deprotonation of stably reduced Q_A [49]. The recovery from the stable state, moreover, is believed to

involve a protein conformational change, since the recovery is inhibited by very low concentrations of the cross-linker glutaraldehyde [49].

In contrast to anaerobic photoinactivation strong illumination in the presence of oxygen leads quickly to irreversible impairment of electron transport and to the subsequent degradation of the D1 protein. By what mechanism does oxygen exert its damaging effect on PS II, and is the toxic effect of oxygen in some way connected to the intermediates trapped under the anaerobic conditions? Oxygen radicals [2] and singlet oxygen [58] formed in PS II under light stress have been suggested to be potential damaging species.

Relevant to the questions raised above was the observation of a spin-polarized chlorophyll triplet signal by EPR spectroscopy in the last three states of the anaerobically photoinactivated PS II membranes [49,51]. In the stable and non-decaying intermediate states such a chlorophyll triplet is consistent with the double reduction and release of Q_A from the reaction centre, respectively, since both events would result in recombination of the primary charge pair $P680^+$-$Pheo^-$. That the triplet signal is found in the semi-stable state, containing single reduced Q_A, would be somewhat surprising, since the negative charge would be expected to lower the yield of charge separation [59]. However, there would be no charge repulsion in the case for a protonated Q_A^- as suggested for the semi-stable inhibition intermediate (Fig. 5).

Chlorophyll triplets by themselves are not harmful but in the presence of molecular oxygen they readily react to produce singlet oxygen [60]. It was therefore of great significance that the chlorophyll triplet was quenched with concomitant induction of D1 protein degradation when oxygen was flushed into anaerobically photoinactivated samples [49]. It therefore seems likely that singlet oxygen formed in the reaction centre will rapidly react with and hence damage components in the vicinity of its formation site. This would explain the selective and irreversible damage to the reaction centre, in particular the D1 protein. It should be noted that illumination in the presence of oxygen induces no accumulation of triplet-forming stably-reduced Q_A species. Once formed under aerobic conditions, these intermediates will be rapidly quenched due to the formation of singlet oxygen, thereby making the photoinactivation irreversible. In turn, this suggests that an empty Q_A-site is not a very likely event during strong illumination in the presence of oxygen.

The primary target for the singlet oxygen is not known but studies on isolated PS II reaction centres by Barber and co-workers have indicated that this very reactive species leads preferentially to destruction of the P_{680} chlorophyll [61]. Whether this is due only to an attack on a chlorophyll molecule or also to one on a ligating histidine, known to readily react with singlet oxygen [62], remains to be established.

Is this detailed mechanism for acceptor-side-induced photoinhibition (Fig. 5) in accordance with other experimental observations concerning this problem? Below we will discuss other pertinent mechanistic data with special emphasis on other in vitro studies.

Setlik and co-workers resolved the photoinactivation process into three kinetically different phases [47,53]. An initial very fast phase, with a typical rise in Fo fluorescence, was only observed under anaerobic conditions. This phase was ascribed to the buildup of a stable Q_A^--species. The following slow phase, which could also be observed in the presence of oxygen and was characterized by the loss of F_m, was suggested to be the result of neutralization of negatively charged Q_A^-, possibly through protonation. The final phase (very slow) was linked to loss of the charge separation reaction and is probably related to degradation of the D1 protein.

Studies on light-induced inactivation of PS II electron transport in cyanobacteria, using fluorescence and thermoluminescence measurements, suggested that the initial inhibition was a block in the electron transfer between Q_A to Q_B, followed by inhibition of electron transport to Q_A [63]. Interestingly enough, inhibition of electron transfer from Q_A to Q_B in a cyanobacterial mutant was also correlated to accelerated turnover of the D1 protein [64,65]. This was explained by the action of damaging free radicals that are produced when electron transfer is inhibited while plastoquinone still is bound to the Q_B site. An alternative explanation in light of various in vitro observations is that inhibition of electron transfer from Q_A to Q_B would lead to stably reduced Q_A species and thus also to the formation of the damaging singlet oxygen as discussed previously.

In another study in isolated thylakoids, based upon thermoluminescence measurements, it was concluded that the primary target of photoinactivation was before the Q_B-binding site and that Q_A and Q_B may undergo simultaneous impairment [66]. In a study of PS II membranes subjected to strong illumination [56] it was shown by EPR that the possibility of forming the Q_A^--Fe^{2+} signal was impaired concomitantly with the loss of steady-state oxygen evolution. The inhibition of the primary charge separation, measured as formation of the pheophytin$^-$ EPR signal, was a fairly late event. This is consistent with flash-induced absorbance measurements of photoinhibited thylakoids performed by Allakhverdiev and co-workers [67]. These observations, combined with the fact that the EPR measurements were performed at temperatures where the Q_A to Q_B transfer is blocked, suggested an inhibition of electron transport due to impairment of the Q_A-function.

There are also several pieces of experimental data emphasizing that the secondary quinone acceptor Q_B is the primary site of photoinactivation [2]. This concept is partly based upon the different degrees of impairment observed when DCMU sensitive and insensitive electron acceptors are used during the activity measurements [41,68–70]. Moreover, thermoluminescence measurements in vivo of photoinactivated *Chlamydomonas* cells have shown that a shift of the B-band, indicative of charge recombination between Q_B^- and the S-state, precedes the loss of the Q-band, representing recombination between Q_A^- and the S-state [2,71,72]. It was concluded that the amount of reducible Q_B decreased faster than that of Q_A.

The mechanism for light-induced impairment of the Q_B site is not known. However, inactivation due to the formation of radicals from the oxidation of the secondary quinone by molecular oxygen [36] or destablilization of Q_B followed by irreversible modification of the D1 protein has been suggested [70,71]. It has even been speculated that such photoinhibition at the Q_B site can lead to increased lifetimes for the highly oxidizing radicals $P680^+$ and/or Tyr_z^+, thereby leading to donor side inactivation [73].

The detailed role of the primary (Q_A) and secondary (Q_B) plastoquinone acceptors during the photoinactivation process is still therefore somewhat controversial and remains to be established, particularly with respect to the situation in vivo (see subsection IV-C).

Apart from singlet oxygen [49,58,61] there are indications that oxygen or hydroxyl radicals might also be involved in damage of the D1 protein during the photoinhibition process [36,69,74]. In several experiments various oxygen radical scavengers have been shown to protect partially the D1 protein from degradation [69,75,76]. Such oxygen radicals could be formed in the PS II reaction centre via the chlorophyll triplet, via Fenton chemistry at the non-haem iron or via semiquinone anions. It remains to be established how specific and frequent such damage to the D1 protein would be in comparison to damage mediated via singlet oxygen formed in the reaction centre [49].

IV-B. Donor-side-induced photoinactivation

Photoinhibition due to a fully-reduced acceptor side, as discussed in the previous section, is generally considered to be the main mechanism for impairment of PS II electron transport and D1 protein damage. However, an additional inactivation process occurs when the donor side of PS II is unable to keep up with the rate of withdrawal of electrons from P680, a situation that may statistically occur during normal steady-state electron transport (Fig. 5). This may lead to the accumulation of long-lived, oxidizing radicals on the donor side which in turn would have a high probability of inducing rapid inactivation of PS II electron transport and protein damage [see 18,43]. In contrast to the acceptor-side-induced inactivation mechanism, which requires high-light conditions, the donor-side induced inactivation mechanism could occur under both low and high light intensities.

The experimental evidence for donor-side inactivation has mainly been obtained from studies on photosynthetic samples where a non-functional donor side of PS II has been deliberately induced prior to the photoinhibitory illumination. Increased susceptibility of PS II to photo-inactivation and/or to protein damage has been shown in hydroxylamine-treated leaf segments [77], in Cl^--depleted inside-out thylakoid vesicles from mangroves [78], in Cl^--depleted thylakoid membranes [79,80], and also in isolated in PS II reaction centre particles [61]. Moreover, it has been shown that the quantum yield of electron transport inactivation increases 1000-fold in Tris-treated PS II membranes ($1 \cdot 10^{-4}$ inhibited reaction centres/quantum) as compared to normal oxygen evolving PS II membranes ($2–3 \cdot 10^{-7}$ inhibited reaction centres/quantum) [44]. PS II photoinactivation and D1 protein degradation are also enhanced in the *Scenedesmus* LF-1 mutant which is unable to evolve oxygen [73] and in *Synechocystis* 6803 deletion mutants lacking the *psbO* gene that encodes the manganese stabilizing 33 kDa extrinsic protein [81,82]. There could also be physiological stress conditions that might reduce the efficiency of the PS II donor side. For example, in plants where a freeze-induced loss of extrinsic proteins involved in water splitting has occurred there is an increased sensitivity to photoinhibition [83].

Donor-side inactivation has been shown to result from impairment of electron transport between the manganese cluster and P680 [44,80,84,85]. More specifically, Blubaugh and co-workers were able to resolve three kinetically different phases of inactivation in hydroxylamine treated PS II membranes using EPR and optical spectrophotometric analyses of $P680^+$ reduction [84,85]. The first event was proposed to be a decrease in the rate of electron transfer between Tyr_z and $P680^+$, followed by a loss of Tyr_z^+ formation. This inhibition was attributed either directly to damage to the tyrosyl residue or to amino acids in the immidiate vicinity. The final, very slow, phase was a loss of Tyr_D^+ formation. The two first events described by Blubaugh and co-workers are supported by the work of Eckert and co-workers [44] measuring photoinactivation in Tris-washed PS II membranes by flash-induced absorption changes at 830 nm which reflects the transient $P680^+$ formation. It was concluded that photoinactivation induced on the donor side was clearly due to impairment of electron transfer between Tyr_z and $P680^+$.

The most likely reason for impairment of electron transport is that continuous illumination generates abnormally stable oxidizing radicals on the donor side of PS II when the electron flow from the manganese cluster is insufficient. Both $P680^+$ and Tyr_z^+, are highly oxidizing and potentially hazardous, and likely to rapidly oxidize nearby pigments, redox components or amino acids as proposed by in Refs. 43,80,85–87, resulting in an inactivated centre. A somewhat more complicated picture was recently presented by Cheniae and co-workers [88], who suggested that minimally two mechanisms underly donor-side photoinactivation of hydroxylamine-treated PS II membranes. The first one being a rapid process requiring both superoxide and cation radical(s) of the PS II reaction centre, while the second one was proposed to be a slower process driven only by cation radicals. As in the case of acceptor-side-induced inactivation of PS II electron transport, the donor-side inactivation also leads to D1 protein degradation, indicative of an irreversible damage to this protein [80,89–91]. Experimentally, this has been studied in Cl^- depleted PS II membranes [80,89] and in isolated PS II reaction centres devoid of the water oxidation system [90]. The targets for these damaging oxidizing species are not known at present, but illumination of purified PS II reaction centre particles in the presence of an artificial electron acceptor leads to an irreversible photobleaching of B-carotene and the accessory chlorophyll designated Chl-670 [87].

It should be noted that for both of the mechanisms for photo-inactivation discussed above, although they have their origins from the acceptor or donor sides, respectively, the final damage to the reaction centre is most probably targeted around the primary electron donor P680. There is also experimental evidence suggesting that under some conditions P680 itself may function as a primary target for photoinactivation [44,92].

Since donor-side inactivation of electron transport does not require high light intensities the associated D1 protein damage and degradation would occur also under low light. This might explain the turnover of the D1 protein seen under quite low intensities in vivo which normally is not related to photoinhibition.

IV-C. On the mechanism of photoinactivation in vivo

As discussed above there is now compelling evidence, mainly based upon in vitro studies, that light-induced impairment of electron transport can occur at both the acceptor and donor sides of PS II (see Table I). However, it is not yet clear what is the dominating mechanism in vivo [2,18,43,44] and additional ways of photoinactivation can not be excluded at this stage. Certainly, most early studies would favour an inactivation at the acceptor side of PS II, targeted to the Q_B-site (see Ref. 2), but considering all the recent mechanistic data (see Ref. 18) particularly the observation of stably reduced Q_A-species and a distinct donor-side inactivation, the issue has become more complicated.

In contrast to the situation in vitro, complete reduc-

TABLE I

Comparison between accceptor- and donor-side-induced photoinhibition of Photosystem II

	Type of inactivation	
	Acceptor-side	Donor-side
Light requirement	High light	High light/low light
Inactivation of electron transfer	Inoperational Q_B-site [2] Accumulation of stably reduced Q_A [49]	Accumulation of $P680^+$ and/or Tyr_Z^+ [44,80,84–86]
Irreversible damage to the reaction centre	Singlet Oxygen [49 58] (oxygen radicals) [36,64,72]	Accumulation of $P680^+$ and/or Tyr_Z^+ [44,80,84–86]
Primary target for damage	P680?	Chl_{670} / β-Carotene? [61]
Conditions for D1 protein degradation	oxygen required [2,40,49] temperature dependant [42] Inhibition by serine protease inhibitors [105,109]	partly proceeding under anaerobic conditions [89–91] temperature dependant [105] Inhibition by serine proteinase inhibitors?
Early D1 protein fragments	*N**-23 kDa [110] *C***-16 kDa [110] C**-10 kDa [111]	*N**-9 kDa [111] *C***-24 kDa [111,118]

* *N*-terminal

** *C*- terminal

tion of the PQ-pool leaving the Q_B-sites inoperational is less likely to occur in vivo. Moreover, the accumulation of Q_A^- is not considered to be a common event [93]and photoinhibition has been reported to occur in vivo even when the majority of the PS II centres contain Q_A in its oxidized form [94]. Still, it is quite arealistic to suppose, that under high-light stress conditions the operational frequency of the Q_B-site would be sufficiently low as to increase the probability of stably reduced Q_A-species to be formed in a small but significant fraction of PS II centres, thereby leading to inactivation and irreversible damage to these reaction centres (Fig. 5). The probability of these events occurring would increase if the high light were combined with other stress conditions such as CO_2-deficiency and low temperatures. If additional events occur during acceptor-side inactivation, such as protein conformational changes in the reaction centre, making the Q_A to Q_B electron transfer less efficient [2,95], the probability of functional impairment at the acceptor-side in vivo would be increased. Experimental support for an acceptor-side-mediated mechanism in vivo comes from the work by Ohad and co-workers indicating that in its initial stage the inactivation can be reversible without de novo protein synthesis [71,72]. Such a reversibility would not be expected if the inactivation was induced from the donor-side. Moreover, fragments typical of acceptor-side-induced D1 protein degradation [91] have been identified under in vivo conditions [3].

It is not very likely that the D1 protein turnover seen at moderate light intensities (see Ref. 3) has its main origin in impairment through the acceptor-side-induced mechanism but rather described due to donor-side inactivation. Still, the donor-side mechanism, has so far only been demonstrated in vitro after various pretreatments to deliberatly impair electron donation to P680 [18], while the mechanism of donor-side photoinactivation in vivo is not known. It is clear, however, that once a reduction in the capacity of electron donation to P680 has occurred the centres would be very sensitive to light and would undergo rapid damage with higher quantum yield than that noted for the acceptor-side-induced mechanism [44,80]. In vivo, it has been speculated that such a reduction in the electron donation to PS II, leading to sensitization for light damage, could be induced by creation of a too low pH in the lumenal compartment [96,97] or due to release of extrinsic proteins at chilling [83] or at elevated temperatures [98], thereby leading to destabilization of the manganese-cluster.

V. Degradation of damaged D1 protein

Once damaged, the D1 protein has to be removed to enable a new copy of the protein to be assembled into the PS II complex in order to reestablish photosynthetic function. Therefore, even though a degradative process, D1 protein depletion should be considered as the first phase in the repair of photodamaged PS II reaction centres. As will be discussed below, the majority of experimental data shows that damaged D1 protein is degraded by a proteolytic process. Important questions to be addressed are the identity and properties of this proteolytic machinery and what targeting or triggering mechanisms enable discrimination between a native and a damaged D1 protein.

As discussed above, the D1 protein turns over at a rate considerably faster than any other PS II subunit [99] although the D2 protein also shows a detectable but slow light-induced turnover [2]

In studies on the mechanism of D1 protein turnover it has been diffucult to discriminate between effects related directly to the degradation and preceding events associated with the inactivation of electron transport and the oxidative damages. Moreover, studies in vivo are to some extent hampered by the simultaneous biosynthesis of new D1 protein and reassembly of functional PS II complexes. We will below describe some quite recent progress towards understanding the mechanism of D1 protein degradation using in vitro studies of isolated thylakoid membranes and various subthylakoid fractions, including PS II core particles and reaction centres. Analysis of the relative content of the D1 protein has been based upon samples prelabelled with [^{35}S]methionine, quantitative western-blotting or atrazine binding [2]. Out of these methods the immunological one appears to be the most specific (Fig. 6), while data based upon atrazine binding should be treated with some care since conformational changes in the Q_B-binding region probably precede the actual degradation.

Early in vitro studies by Ohad and co-workers on isolated pea and *Chlamydomonas* thylakoids demonstrated disappearance of the 32 kDa Q_B-protein during photoinactivation of electron transport [41,100]. It was concluded that the D1 protein was degraded by a highly specific proteinase intrinsic to the thylakoid membrane and that targeting of the protein for degradation involved a conformational change. Still, the problem of these early in vitro studies was that one merely observed a disappearance of the D1 protein and a direct connection with breakdown fragments could not readily be made. At the same time, high molecular weight aggregates were observed during SDS-PAGE analysis of strongly illuminated thylakoids, which contained several PS II subunits in addition to the D1 protein [2,101]. It could therefore not be excluded that this aggregation was the explanation for the light-induced disappearance of the D1 protein seen after the electrophoretic analysis. The in vitro approach was therefore conceptually abandoned for a period and it was thought that D1 protein degradation

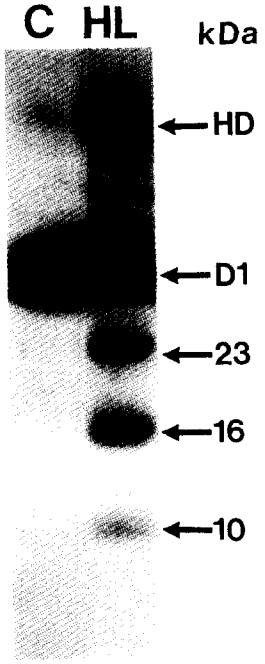

Fig. 6. Immunoblot demonstrating light-induced D_1 protein degradation and fragment formation in PS II core complexes. Illumination was performed under strong light (5000 $\mu E m^{-2} s^{-1}$) at 25°C at a chlorophyll concentration of 100 $\mu g\ ml^{-1}$. Lane C shows a control kept in darkness and lane HL the sample illuminated for 30 min. The proteins were subsequently separated by SDS-PAGE, electroblotted onto a PVDF membrane and immunodecorated with D_1 protein antisera and ^{125}I-protein A. HD: D1/D2 protein heterodimer. Fragment sizes are indicated.

could only be analyzed using in vivo systems. However, a new generation of in vitro photoinhibition studies using quite simple PS II preparations, overcame many of the experimental concerns mentioned above [18,43]. Most of all, it was possible to create experimental conditions under which the light-induced disappearance of the D1 protein could be correlated to the appearance of breakdown products and that the process could be controlled not only by light but also by variations in temperature and oxygen content.

What are the mechanistic implications from in vitro studies of D1 protein degradation? First of all, can the degradation of damaged D1 protein be considered as an all-proteolytic event? As discussed in Section IV, the light induced inactivation of electron transport can give rise to highly reactive intermediates which may in turn cause a direct cleavage of peptide bonds in the D1 protein. This could, for example, occur through modification of proline residues [102] or through scission of peptide bonds by reactive oxygen species [103,104]. Naturally, it is not conceivable that the whole 32 kDa polypeptide chain could be cut into small peptides or even amino acids by such 'photocleavages', but they could be responsible for the initial cleavages of the D1 protein. However, at present the majority of experimental evidence strongly favours an all enzymatic digestion of the D1 protein in particularly in connection with acceptor-side-induced photoinactivation. The evidence can be summarized as follows; (i) the kinetics of inactivation and D1 protein degradation are not the same (ii) D1 protein degradation occurs in complete darkness at room temperature, once subjected to strong illumination in the cold (iii) proteinase inhibitors can give over 50% inhibition of the degradation.

In the case of donor-side-induced degradation there also appears to be proteolytic degradation. The kinetics of the disappearance of D1 protein lag behind the inhibition of electron transport also in Cl^--depleted thylakoids [80] and in isolated PS II reaction centres [105]. Moreover, there are different pH optima for the inactivation and degradation events and the latter can partially be blocked by proteinase inhibitors [105]. A degradation of the D1 protein based upon proteolysis is in accordance with numerous other biological systems where oxidative stress to protein occurs and the repair process requires proteolysis and resynthesis of the damaged proteins [103].

The identity and characteristics of the proteinase or proteinases involved in the efficient digestion of the D1 protein are not known at present, but some progress has been made in the last few years [18,43]. As revealed from studies in several research groups, D1 protein degradation can easily be demonstrated after photoinhibitory illumination of isolated thylakoids [28,41,80,106] and purified PS II grana membranes [46,47,107]. The indentification of proteolytic breakdown products is not easy in these membrane systems, although fragments of the D1 protein similar in size to those seen in vivo [3] can be identified [42]. This implies that the further proteolysis of the primary breakdown fragments of the D1 protein is at least as efficient as the initial proteolytic events.

An important observation was that significant amount of D1 protein degradation with high recovery of proteolytic fragments, could be obtained in photoinactivated PS II core preparations (Fig. 6) [91,108–110] and isolated PS II reaction centres [90,105,111]. These results suggest that the proteinase(s) responsible for the initial steps of the D1 protein degradation process is an integral part of the PS II complex itself. The proteolytic activity appears to be present in stoichiometric rather than catalytic amounts. This is supported by the substantial light-induced degradation of the D1 protein that can be observed in the isolated particles, even after dilution. This suggests a close interaction between enzyme and substrate with at least one proteinase per PS II complex [110]. Possibly only the early digestion steps are catalysed by proteinase(s) tightly associated with PS II. As can be seen in Table II, only the primary proteolysis takes place in highly purified PS II sub-core particles and in isolated reaction cen-

TABLE II

D_1 protein degradation in various system ranging from whole plant to isolated PSII reaction centers

	D_1 protein			
	Primary cleavage	Total proteolysis	Detection of D_1 protein fragments	Refs.
In vivo	+ +	+ +	*	3
Isolated thylakoid membranes	+ +	+ +	*	42,106,115
PSII enriched membranes	+ +	+ +	*	[a]
PSII core complexes	+ +	+	**	108
Highly purified PSII core particles	+ +	−	**	91,110
PSII reaction centres	+	−	*	90,105,112

+ +; Fast,
+; Slow
−; Undetactable
*; Low amounts
**; High amounts

[a] (Salter, unpublished)

tres. Complete proteolysis requires a more intact, membraneous preparation [112].

The initial D1 protein degradation can to a large extent be inhibited by proteinase inhibitors [100,105, 109,110]. The most potent group of inhibitors are those effective against serine-type proteinases [109]. This includes the taxonomic serine proteinase inhibitor diisopropyl fluorophosphate (DFP) [103], which covalently binds to the presumed activated serine of the catalytic site of the proteinase [113].

The pH optimum for the proteolysis is in the range pH 7.5–8.0 [100,105,110]. The activity is stimulated by Mg^{2+} ions but the proteolysis readily proceeds in the absence of this ion [110]. Moreover, the degradation does not require the presence of ATP which excludes ATP-mediated routes for D_1-proteolysis such as the ubiquitin pathway.

Conclusive identification of a 'D_1-proteinase' associated with the PS II complex has not yet been made. It has been suggested that the degradation could be autoproteolytic [90,108]. In line with this suggestion is the observation of D1 protein degradation and fragment formation in isolated PS II reaction centre particles [90,105,111,112] which are composed only of the D_1- and D_2-proteins, cytochrome *b*-559 and the *psb*I-gene product [22,23]. Another experimental result suggests that the chlorophyll *a*-binding protein CP43 harbours a proteolytic capability. This protein was shown to specifically bind the radiolabelled serine-proteinase inhibitor DFP, which is an effective inhibitor of D1 protein degradation in isolated PS II core particles [110]. However, the apparent lack of similarity to known serine proteinases [113] and the presumed absence of CP43 from isolated reaction centre particles, which are able to degrade the D1 protein, would speak against CP43 as the proteinase involved. On the other hand, the amount of CP43, often a contaminant of PS II reaction centre preparations, has so far not been quantified and correlated with the degree of D1 protein degradation in photoinhibition experiments with reaction centre particles. The final identification of the proteolytic 'D1 proteinase' therefore needs further experiments, and continues to be a very central topic. Maybe there is more than one proteinase activity required for degradation of the membrane spanning D1 protein [43,50].

Characterization of the proteolytic fragments obtained in the various PS II preparations would offer a way to determine the primary cleavage site(s) of the D1 protein. So far, however, there has been no success in obtaining direct sequence information from the digestion fragments and it has not been possible to make any detailed conclusions concerning their origin in the primary sequence of the D1 protein. Still, some indirect data are available. Strong illumination of isolated PS II core complexes which have a functional water-splitting complex and consequently are undergoing acceptor-side induced photoinactivation, give rise to 23, 16 and 10 kDa fragments (Fig. 6) and occasionally to 14 and 13 kDa fragments [91,108]. Analysis using site specific antibodies and specific radiolabelling of amino acids suggests that the 23 kDa fragment is of N-terminal origin whereas the 16 and 10 kDa fragments are of C-terminal origin [91,108]. The kinetics of the fragment appearance suggests all three fragments to be early digestion products [108]. Mapping studies on wheat PS II core complexes advocate that the N-terminal 23 and C-terminal 10 kDa fragments are generated from one cleavage site [91]. This would imply a primary proteolytic cleavage in the loop exposed at the outer thylakoid surface that connects transmembrane helices D and E of the D1 protein (Fig. 2). This observation would be consistent with previous theoretical and experimental suggestions on the primary site of D1 protein degradation (see Ref. 99). A 23.5 kDa fragment was identified in addition to fragments in the 8–14 kDa molecular mass range during D1 protein turnover in vivo [3]. Proteolytic mapping of the 23.5 kDa fragment suggested a cut-site in the stromal D-E loop. Additional evidence for such a location comes from work of Trebst and co-workers [114,115] who observed an 8 kDa fragment following ultraviolet illumination of thylakoid membranes. Sequence analysis of the N-terminus of this fragment suggested a cleavage site at Phe-239. The work of Barber and co-workers suggests a cleavage site about 1 kDa further to the C-terminus [91,116]. In addition to the possible cleavage site, the D-E loop of the D1 protein contains a region resem-

bling a PEST sequence [3,99]. Proteins carrying such a sequence are normally susceptible to degradation [117]. This sequence in the D1 protein, which is conserved from procaryotic cyanobacteria to higher plants is located between Arg-225 and Arg-238. Although the hypothesis of a role for a PEST sequence in D1 protein turnover is challenging, there is so far no experimental support for such a possibility.

Studies on D1 protein degradation in isolated PS II reaction centres [90,105] have suggested another primary cleavage site, located in the loop exposed to the thylakoid lumen that connects transmembrane helices A and B [111] (Fig. 2). This suggestion was based upon the fragment pattern obtained in these non-oxygen-evolving PS II particles. C-terminal 24 and 16 kDa fragments were obtained in addition to an N-terminal 10 kDa fragment. A third cleavage site has been proposed by Barbato and co-workers, who suggested a cleavage site in the lumenal hydrophilic loop connecting transmembrane helices C and D [118]. This suggestion was also based upon the fragment pattern obtained in PS II preparations with a non functional donor side. One explanation for these apparently contradictory results has come from photoinhibition studies on isolated PS II core complexes with functional or impaired water-oxidation system [91]. The fragments identified in the latter case resemble very much those obtained in isolated reaction centres consistent with a lumenal primary cleavage site at the lumenal side of the membrane. In contrast, strong illumination of intact oxygen evolving PS II core complexes gave the typical fragment pattern consistent with a 'traditional' primary cleavage in the stromally exposed D-E loop. Thus, the primary cleavage site may be located at different sides of the thylakoid membrane depending on whether the D1 protein degradation is induced by acceptor-side or donor-side photoinactivation [91] (see Table I). Still, it must be realized that this distinction can only include the primary cleavages since complete removal of the damaged D1 protein most probably requires endoproteolytic cuts at both sides of the membrane in the light of its transbilayer organization. Therefore, as mentioned above, more than one proteinase may be required for the efficient degradation of the D1 protein.

Irrespective of the identity of the proteinase(s), there must be some kind of discrimination between native and damaged D1 protein. It is clear from the photoinhibition studies of thylakoid membranes kept at low temperatures that the damage itself does not lead to degradation [42,119] and some additional triggering event appears to be required to turn the D1 protein into a substrate for proteolysis. The exact molecular basis of such triggering is not known at present. The early suggestion of a conformational change in the D1 protein once it has become damaged [41] still appears to be the most plausible [120], but covalent modifications prior to degradation cannot be excluded (see Ref. 2).

There is some evidence in support of a conformational change. One line of evidence can be derived from the partial protective effect of DCMU on D1 protein degradation seen both in vitro [39,46,106] and in vivo [2,38,76,121]. This protective effect is quite specific and is a property of urea and triazine-type herbicides while phenol-type herbicides are less efficient inhibitors of degradation [39,46,122]. Since the first group of herbicides binds firmly in the Q_B-pocket of the D1 protein they may restrict a conformational change that is required to turn the damaged protein into a substrate for proteolysis. Support for this hypothesis has recently been observed [123]. It was shown that DCMU added to thylakoids after photoinhibitory illumination in the cold but prior to warming up the sample (see Ref. 42) showed the normal protective effect, while no reduced rate of D1 protein degradation was seen if the herbicide was added after the temperature rise.

A gross conformational change of photodamaged D1 protein is also suggested by the release of manganese from the PS II reaction centre prior to proteolysis [119]. Moreover, alterations in the conformation of photodamaged PS II reaction centre particles have been detected by Barber and co-workers using FTIR-spectroscopy [124]. Similar alterations were seen after treatment of reaction centre particles by certain detergents. It can therefore be speculated that conformational changes induced by other means than photoinhibitory treatment can lead to triggering and D1 protein degradation. Indeed, in thylakoids heated to 40°C in complete darkness, loss of D1 protein has been observed in preliminary experiments (Sundby et al. unpublished data).

Among the hypotheses concerning a covalent triggering of the D1 protein for degradation, the most likely one is that involving the formation of an internal crosslink, possibly through formation of a bityrosine as suggested by Ohad and co-workers [2]. Even though this is a quite attractive possibility and bityrosine formation can occur in proteins exposed to free radicals [104], its verification needs further experimental analysis. Covalent phosphorylation of the D1 protein has also been inferred as a step in the degradation process [125]. As will be discussed in Section VII, there is now evidence that phosphorylation of the D1 protein is not required for the triggering process. In fact, in vitro studies suggest that phosphorylated D1 protein is a relatively poor substrate for degradation [110,126].

The other subunit of the PS II reaction centre heterodimer, the D2 protein, is also degraded during photoinhibitory conditions both in vivo [127] and in vitro [28,108,115] and in vitro. D2 protein fragments in

the range of 19–29 kDa have been identified [90,101,108]. However, compared to degradation of the D1 protein, the process is considerably slower. At present we do not know whether there is a direct photodamage of the D2 protein or if its degradation is caused by secondary proteolysis due to disassembly processes in the PS II complex once the D1 protein has been degraded.

Prolonged illumination of PS II preparations also leads to degradation of the chlorophyll *a* binding proteins CP43 and CP47 [47]. Loss of CP43 [128] has also been seen in other photoinhibition studies. It is not likely that extensive degradation of the proteins occurs in vivo, since they do not show high turnover in pulse chase experiments [127].

There are no reports that the L and M subunits of photosynthetic purple bacteria reaction centres undergo a light-induced turnover, despite their functional and structural homology with the D_1 and D_2 proteins of PS II (see Ref. 2) This does not neccessarily mean that there is no photoinactivation in these organisms under conditions of excess light. Indeed, photoinactivation processes, that involve the formation of doubly reduced Q_A, have been inferred from FTIR-spectroscopy of bacterial reaction centres [129]. In contrast, the inactivated state may not lead to any irreversible damage to the reaction centre, since the oxidation potential of the bacterial triplet would make it less likely to produce singlet oxygen. In addition, the normal environment of the purple bacteria is anaerobic. The reaction centre chlorophyll has a more moderate oxidizing potential (0.5 V) as compared to that of plant PS II, thus reducing the probability of donor-side-induced damage. Even if damage to the L and M subunits should occur, the survival of the rapidly dividing bacterial population may not rely on the repair of individual reaction centres.

VI. Secondary changes and lateral movements of the Photosystem II complex related to D1 protein degradation

Considering the molecular organization of the PS II reaction centre once it loses the D1 protein, other secondary changes are also likely to occur to the complex. Release of the three extrinsic polypeptides (33, 23 and 16 kDa) from the inner thylakoid surface into the lumenal phase is one consequence of the D1 protein degradation (Fig. 1) [28,107]. Four Mn atoms are also released for every D1 protein triggered during the course of the degradation process [107]. Apart from the Mn atoms, the D1/D2 protein heterodimer ligates chlorophyll *a*, pheophytin, carotenoids, plastoquinone and a single atom of non-heme iron (Figs. 1 and 2) [22]. As discussed above, several of these cofactors are lost or damaged during earlier stages of the photoinhibitory process. The fate of the other cofactors is not known, but their release from the PS II complex can be expected to be a consequence of the D1 protein degradation.

Moreover, studies on the organization of PS II following strong illumination in vitro have indicated a partial disassembly of the hydrophobic core of PS II [107]. At present, it is not clear to what extent this disassembly occurs in vivo. Nevertheless, these additional changes point to the problem that repair of photoinhibition involves not only biosynthesis and integration of the newly synthesized D1 protein but also religation of cofactors and assembly of the protein subunits.

Plant thylakoid membranes show a unique lateral heterogeneity in their organization (see Ref. 9). PS I and the ATP synthase are located only in the non-appressed thylakoid membrane regions and are laterally segregated from the majority of PS II and LHCII (PS IIα) which are located in the appressed membranes. Only a minor pool of PS II complexes with small LHCII antennae (PS IIβ) are present in the stroma exposed thylakoid regions [130]. The cytochrome *b/f* complex is the only protein complex that is equally distributed between the two thylakoid regions (see Ref. 9). Laterally segregated protein complexes are functionally connected by rapid diffusion of the small electron carriers, plastoquinone and plastocyanin (see Refs. 58,131).

This lateral heterogeneity poses certain problems for the repair of photodamaged PS II complexes. The damage occurs mainly in the appressed thylakoid membranes [107,132–134], where the PS IIα centres with large LHCII antenna are located. Conversely, newly-synthesized D1 protein is inserted into the thylakoid membrane in stroma-exposed regions [135,136] to which the ribosomes have access. This raises the question of whether it is only the D1 protein that migrates laterally along the thylakoid membrane or whether the comigration of other subunits is also required in the repair of functional PS II.

Such lateral movements of protein complexes between appressed and non-appressed thylakoid regions are known to readily occur under various physiological conditions. Upon phosphorylation of the LHCII, it dissociates from PS II and migrates to the PS-I-rich non-appressed thylakoid regions in a process thought to be essential for regulating the light-harvesting process (see Refs. 9,137). Moreover, lateral rearrangements are also known to occur at elevated temperatures [138,139]. In addition, and directly connected to the problem addressed above, is the observation that new PS II proteins, both of plastid and nuclear origin, once inserted into the stroma exposed thylakoids, diffuse into their functional site in the appressed thylakoid regions [135,136,140].

Accumulating evidence supports the hypothesis that there is lateral migration of several PS II subunits during the degradation and resynthesis of D1 protein [107,134,141,142]. Thylakoid subfractionation experiments following photoinhibitory treatment reveal elevated amounts of PS II subunits in the stromal thylakoid fraction, while most of the LHCII appears to remain in the granal fraction. To what extent also dissociation of individual PS II core subunits from the complex occurs is not clear at present. Experiments with isolated thylakoids indicate partial disassembly of the PS II complex [107]. The increase of the various PS II subunits in non-appressed membranes is not uniform, suggesting that the photodamaged PS II does not leave the appressed membranes as one entity. Disassembly of PS II core polypeptides during in vitro photoinhibition is in accordance with results from studies of PS II assembly in different cyanobacterial and *Chlamydomonas* mutants [143,144], which have clearly demonstrated that stable assembly of PS II complexes in vivo is not possible in the absence of D1 polypeptide.

In vivo, the degree of PS II disassembly associated with D1 protein degradation is still harder to judge. However, both experimental data and theoretical considerations suggest it may be more limited.

According to the experiments of Melis [142], only peripheral LHCII detaches from the PS II complex and the photodamaged PS IIβ-like centres, composed of the PS II core plus the internal LHCII antenna, leave the appressed membranes. Photoinhibition of intact *Chlamydomonas* cells [134] and subsequent fractionation of the thylakoid membranes from them demonstrated translocation of PS II core complexes, still containing assembled D1, D2, cytochrome *b*-559 and the 47 kD polypeptide, from appressed to stroma-exposed regions. Other thylakoid subfractionation experiments indicate the migration of only the reaction centre complex, composed of D1/D2 heterodimer and cytochrome *b*-559 with possibly some attached LHCII [141], suggesting some limited disassembly of the PS II core.

The major difference between the experiments with isolated thylakoids and in vivo studies is that in the latter the new D1 protein can be rapidly available. In fact, light stress of intact leaves does not generally lead to a net loss of the D1 protein from the thylakoid membranes although there is significant D1 protein turnover [145,146]. Moreover, all the newly synthesized D1 protein seems to be rapidly assembled into PS II complexes since no free pools of D1 protein can be detected in the thylakoid membrane [134]. Still, it seems likely that limited disassembly of the PS II complex would facilitate the insertion of new D1 protein into the multisubunit PS II complex. Such a limited disassembly may be necessary for religation of cofactors lost or damaged during the various stages of the photoinhibition process. It has been suggested that the modified PS II complex, after translocation to non-appressed thylakoid domains, functions there as an acceptor for precursor D1 protein, and that the rate of recovery would be eventually controlled by the cycling of PS II reaction centre complexes between appressed and nonappressed membrane regions [134].

Melis (see Ref. 142) has also postulated that PS IIβ centres, located in non-appressed membrane regions, function as a reserve pool that under physiological conditions is ready to rapidly replace the photodamaged PS IIα centres in the appressed thylakoid membranes. PS IIβ centres are not susceptible to photoinhibition [132,133], although they are apparently not active in electron transport from Q_A to Q_B (Q_B-nonreducing centres). Upon translocation to the appressed membrane regions, however, they become activated (Q_B-reducing centers), by a mechanism not yet fully understood [147]. According to this model, the pool of PS IIβ centres is maintained in the non-appressed thylakoid membranes in order to function as a first aid in replacing photoinhibited PS IIα centres [142,148, 149].

VII. Protein phosphorylation and regulation of D1 protein degradation

As discussed above, the rate of D1 protein degradation is not only determined by the rate of PS II photodamage but is also under the control of some regulatory mechanism. This mechanism seems to keep the rate of D1 protein degradation in balance with the insertion of newly synthesized D1 protein into the thylakoid membrane, and possibly thus avoids total disassembly of the PS II complex. Indeed, evidence is accumulating indicating that photoinhibited PS II centres preserve their photodamaged D1 protein until it can be replaced by a newly synthesized one [145,146]. Chronic photoinhibition of *Dunaliella salina* has been shown to lead to the accumulation of photodamaged PS II centres that still contain the D1 protein [150]. As will be discussed below, such a retardation of D1 protein degradation may be of physiological significance. Massive repair of damaged PS II is probably not always essential under high light conditions and photodamaged centres may even serve a role in protection against overexcitation of neighbouring active PS II centres.

What might be the mechanism that retards D1 protein degradation? The proteinase itself is an integral component of the PS II complex and is known to be functional even in isolated PS II core and reaction centre preparations [90,105,108,110]. It has recently been reported that illumination of leaves induces the appearance of a modified form of the D1 protein,

designated 32* or D1* (Fig. 7A) [125,126,145,151]. D1* is present only in the appressed membrane regions [125,145] where photoinhibition is likely to occur [132,133]. The proportion of D1* as a fraction of total D1 protein is dependent on light intensity [126,151], suggesting that D1* might be present in photoinhibited PS II centres that contain damaged D1 protein. In vitro photoinhibition experiments with isolated thylakoids, however, indicated that this modification is not a prerequisite for D1 protein degradation [126]. No transformation of the D1 protein to the D1* form occurred when either acceptor- or donor-side induced D1 protein degradation was induced.

Transformation of D1 protein to D1*, however, can be induced in non-photoinactivated thylakoids in vitro under conditions favouring protein phosphorylation (Fig. 7B) [126,151]. It can therefore be concluded that the D1* form seen during electrophoresis is identical to the phosphorylated form of the D1 protein. After such treatment most of the D1 protein is in the D1* form, and still the thylakoids maintain nearly fully active PS II electron transport [126].

It is well established that D1 protein is indeed one of the phosphoproteins of PS II [152,153]. Phosphorylation takes place at the threonyl residue at the N-terminus of the D1 protein which is exposed to the stromal surface of the thylakoids [154]. Recently, it was

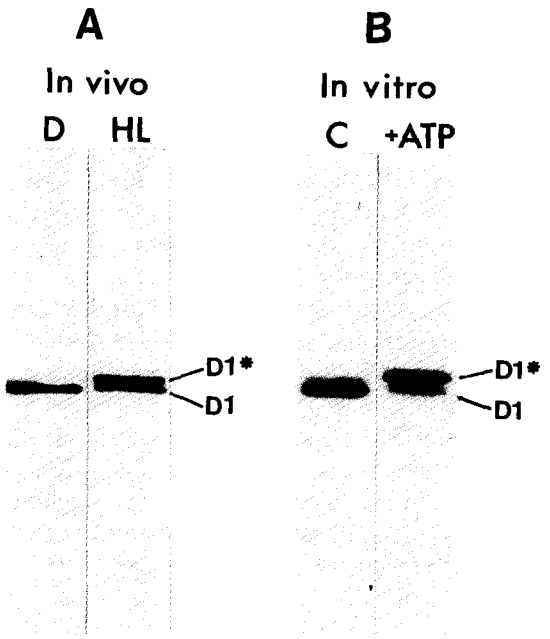

Fig. 7. Immunoblots demonstrating (A) light-induced modification of the D1 protein in vivo and (B) phosphorylation-induced modification in vitro. In A, thylakoid membranes were isolated from dark-adapted pumpkin leaves (lane D) and from leaves illuminated at 2500 $\mu E m^{-2} s^{-1}$ for 3 h (lane HL). In B, control thylakoids (lane C) and thylakoids phosphorylated by illumination at 200 $\mu E\ m^{-2}\ s^{-1}$ for 20 min in the presence of 0.4 mM ATP (lane + ATP). Western blotting was performed according to Ref. 145.

demonstrated that phosphorylation of the D1 protein occurs identically both in vivo and in vitro [151].

A crucial observation was that phosphorylated D1 protein (D1*) is less susceptible to degradation than the unphosphorylated D1 protein during illumination of isolated thylakoid membranes [126] and PS II core particles [110]. This observation, combined with the fact that in higher plants D1 protein degradation seems to proceed via a phosphorylated form [125,145], suggests that protein phosphorylation is involved in D1 protein degradation (Fig. 8). However, it must be kept in mind that phosphorylation is not a prerequisite for D1 protein degradation even in vivo, since in many lower photosynthetic organisms light-induced rapid turnover of the D1 protein readily occurs without protein phosphorylation (Ref. 155; Rintamäki et al., unpublished data). This raises the question of whether, in higher plants, dephosphorylation transiently precedes the final degradation of the D1 protein.

Since D1 protein synthesis occurs only on the stroma-exposed membranes [135,136], while phosphorylated D1 protein accumulates in the appressed membranes [125,145] it is possible that the final degradation in vivo occurs only after the entrance of the damaged PS II complex into the stroma exposed membranes or during the migration from the grana to the stroma exposed regions (Fig. 8). Also, the possibility that partial destacking at the granal margins occurs can not be excluded. Consistent with these considerations, the degradation fragments of the D1 protein after in vivo photoinhibition have been found mainly in stroma-exposed thylakoid regions [141,156].

It could be postulated that phosphorylation allows for coordinated degradation and biosynthesis of the D1 protein during the repair of photodamaged PS II centres (Fig. 8). It should be noted that the CP43 subunit, which is possibly involved in D1 protein degradation [110], also becomes phosphorylated in the light [152,153]. However, it is not currently known whether this has any significance with respect to D1 protein turnover.

VIII. Repair of photodamaged Photosystem II centres

Even though light is the underlying cause of inactivation of electron transport and irreversible protein damage, light is also required to regain a functional PS II. It is well known that only limited repair of photodamaged PS II complexes occurs in darkness and that light is a prerequisite for maximal recovery from photoinhibition [7,37,157]. The light-dependent steps in the synthesis of the D1 protein and in the final assembly of functional PS II complexes still remain to be established. Evidently, from the energetic point of view the process of protein synthesis is costly and requires ATP. However, the light requirement of chloroplast protein

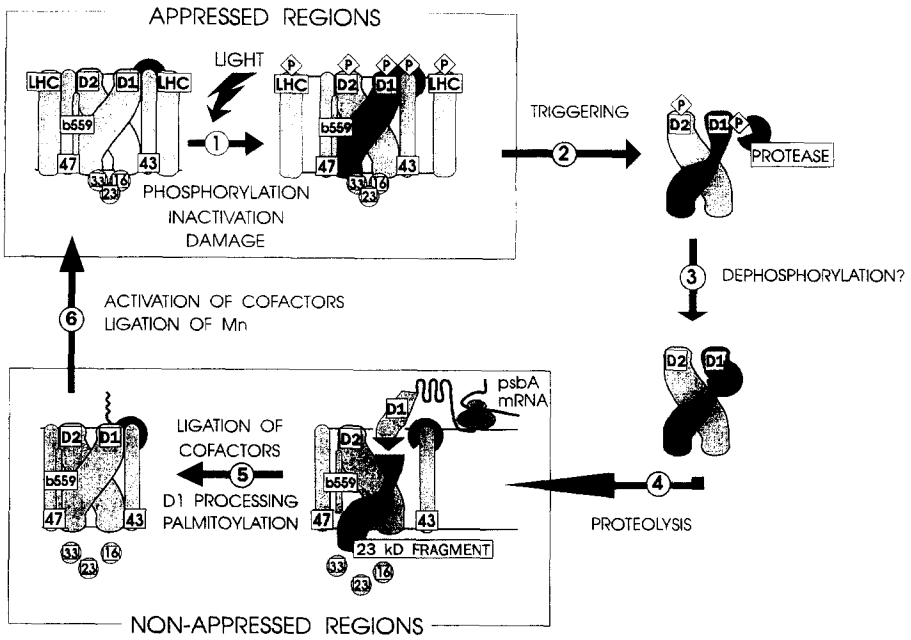

Fig. 8. A hypothetical schematic representation of the association of different phases of the repair cycle of PS II with appressed and non-appressed thylakoid regions in chloroplasts of higher plants. The phases that are known to occur in appressed and non-appressed thylakoid regions are enclosed in boxes. The exact loaction and order of the reactions shown outside the boxes remain to be elucidated. For clarity, only the two heterodimer proteins of the PS II complex are shown. Illumination of leaves results in phosphorylation of most of the PS II proteins in appressed thylakoid membranes (1). These centres are probably those that become photoinhibited under strong illumination (1). Conformational change in the damaged D1 protein (2) exposes the cleavage site. Phosphorylated D1 protein, however, is a poor substrate for the proteinase and dephosphorylation is probably required (3) prior to the primary proteolytic cleavage (4). Final depletion of the D1 protein occurs in the non-appressed thylakoid regions where the 23 kDa degradation fragment is further digested. Concomitantly, the new copy of the D1 protein is synthesized and inserted into the thylakoid membrane. After ligation of cofactors and transient palmitoylation of the D1 protein (5), the PS II complex migrates back to appressed thylakoid regions (6) where finally assembly with the rest of the PS II proteins and activation of water oxidation and electron transport occur.

synthesis is not explained simply by the need for ATP [157,158]. Since recovery from photoinhibition is dependent on the de novo synthesis of D1 protein and on the synthesis and ligation of different cofactors, light regulation may be looked for in any stage of the repair phase (Fig. 4), from the transcription of the *psbA* gene coding for the D1 protein to the functional assembly of the PS II complex in the appressed thylakoid regions (Fig. 8).

VIII-A. Biosynthesis of the D1 protein and ligation of cofactors

In the early literature the *psbA* gene was termed a 'photogene', a gene whose transcripts increase substantially during light-induced chloroplast development [159]. Therefore, it was suggested that regulation of *psbA* gene expression operates at the level of transcription. The light control of cyanobacterial *psbA* gene expression indeed seems to operate at the level of transcription [160,161]. In algae and higher plants, however, there appears to be a post-transcriptional control of *psbA* gene expression [162–164]

The synthesis of D1 protein after photodamage is apparently not limited by the availability of *psbA* transcripts, since in mature leaves these transcripts are rather stable and abundant in the chloroplast stroma [165,166], and show no diurnal fluctuations [167]. Moreover, evidence is now accumulating to indicate that nuclear-encoded protein(s) are involved in stabilizing the *psbA* mRNA through interaction with inverted repeat sequences at the mRNA 3′ end [168].

D1 protein synthesis occurs on ribosomes attached to the stroma-exposed regions of the thylakoid membranes [135,136,169]. Most *psbA* transcripts have been found in the non-polysomal fraction of the chloroplast soluble phase [170]. However, illumination induces the recruitment of *psbA* transcripts into chloroplast polysomes [170] and also increases the attachment of *psbA* mRNA to the thylakoid membranes [171]. No D1 protein synthesis occurs in darkness [162,172].

There is evidence from the green alga *Chlamydomonas* that a single nuclear gene product has an effect on D1 protein translation [173]. Recently, a 47 kDa nuclear-encoded protein has been identified as a light-regulated activator of *psbA* mRNA translation [174]. The binding of this protein to the 5′ end of *psbA* mRNA is light-dependent and supposed to regulate the translation of the D1 protein.

The D1 protein is targeted to stroma-exposed thyl-

akoid membranes early in its synthesis [170]. Binding to the thylakoids may occur via the N-terminus of the D1 protein, since membrane attachment occurs prior to the state when the first hydrophobic α-helix (Fig. 2, helix A) has emerged from the ribosomal tunnel [175]. PS II complexes with damaged or already depleted D1 protein located in stroma thylakoids may function as a receptor for this emerging D1 protein (Fig. 8). This model is supported by the fact that no free pool of D1 protein exists in the thylakoid membrane [134] and that the synthesis and degradation of the D1 protein during repair of photoinhibition seem to be well coordinated [145,146].

The next step in the synthesis and stable incorporation of the D1 polypeptide into the thylakoid membrane has been suggested to involve a light-dependent transformation of protochlorophyllide *a* to chlorophyll *a* [176]. In the absence of chlorophyll no full-length D1 protein has been found to be synthesized but a series of 15 to 25 kDa translation intermediates have been detected. Co-translational binding of chlorophyll *a* to these intermediates ranging from α-helices A to D (Fig. 2) was suggested to stabilize the nascent D1 polypeptide and consequently allow accumulation of mature D1 protein to occur. These 15 to 25 kDa D1 protein translation intermediates have been shown to be associated with polysomes [175]. Furthermore, it has been elegantly shown that ribosomes pause at discrete sites that are consistent with the observation of D1 protein translation intermediates during pulse-labeling. This leads to a model for assembly whereby the D1 protein is inserted into the thylakoid membrane in a segmental manner. Discontinuous translation of the D1 protein may also be important for co-translational binding of other cofactors to the D1 protein besides chlorophyll *a* (e.g., pheophytin, β-carotene and Fe^{2+}) [175].

The phases of D1 protein translation and ligation of cofactors described above are mainly based on studies of rapid protein accumulation upon illumination of etiolated leaves. The same reactions probably also occur in mature leaves during repair of PS II photoinhibition, though under these conditions the use of pre-existing components could be envisaged.

VIII-B. Post-translational modifications of the D1 protein and the activation of electron transport

D1 protein is synthesized as a higher molecular weight precursor of 33.5 kDa [177]. Ohad and coworkers [134] have presented evidence that the D1 protein precursor is indeed integrated into the PS II complex during the repair of photodamaged PS II. The precursor is then rapidly processed at the C-terminal end [169,178] by a specific proteinase [26,27,179] to generate the mature 32 kDa D1 protein.

C-terminal processing of the D1 protein is a prerequisite for another light-dependent step in the reestablishment of functional PS II, i.e., the religation of the released manganese atoms on the lumenal side of PS II [26–28]. This concept is based on a mutant (LF-1) of *Scenedesmus obliquus* that lacks the proteinase and is consequently unable to ligate the Mn atoms [26,27]. Photoactivation of Mn binding and oxygen evolution is driven by at least two successive photoreactions of PS II (see Ref. 180). The full activation of water oxidation requires further rebinding of the released extrinsic polypeptides, 33, 23 and 16 kDa, which are stably maintained in the thylakoid lumen during D1 protein turnover (Fig. 8).

Before PS II can be found in a fully operational state in the appressed membranes, a transient, light-dependent D1 protein acylation by palmitic acid has been reported to occur in stroma thylakoid regions (Fig. 8) [136]. It remains to be established whether this transient acylation step is required to target the migration of the newly repaired PS II complex back from non-appressed to appressed membrane regions [136]. The recovery cycle is finally completed when the repaired PS II core complex attaches to the light-harvesting complex in the appressed thylakoid membrane.

IX. Protective mechanisms against light stress

Photoinhibition of PS II may become a severe problem for plants in field conditions. In order to minimize photoinhibitory damage nature has evolved several mechanisms that serve to protect PS II under potentially damaging light conditions. One of the most important mechanisms is the capability of plants to dissipate excess excitation energy as heat. This phenomenon is related to a creation of a proton gradient across the thylakoid membrane (see Ref. 93) and probably also to the formation of zeaxanthin (see Ref. 12). Phosphorylation of the LHCII complex also serves to divert excess excitation energy away from PS II (see Ref. 18). Moreover, a 'futile' cyclic electron flow around PS II has been suggested to be inducible under high light conditions and hence protect PS II against photoinhibition [86,87]. Once the capacity of the protective mechanisms and the repair cycle of PS II are exceeded, net photoinhibition of PS II takes place and the accumulation of photodamaged PS II centres will occur. The distinction between photoinhibition and photoprotection is, however, not always unambiguous [94,181, 182,183]. Under conditions where the capacity of the repair cycle is limiting, the photoinhibited PS II centres, which are still efficient quenchers of excitation energy, may by themselves serve a protective role and prevent fully irreversible photooxidative damage to the thylakoid membrane.

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