

# ATP and light regulate D1 protein modification and degradation

## Role of D1\* in photoinhibition

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Received 7 November 1991; revised version received 22 November 1991

We have recently shown that during *in vivo* photoinhibition the D1 protein is degraded via a modified form, designated D1\*. Depending on light conditions, the amount of D1\* varies in leaves between 0 and 50% of total D1 content. By isolating thylakoids from leaves acclimated to different light levels, and performing photoinhibition experiments on these thylakoids, the following results on D1 protein degradation were obtained: (i) the protease involved in D1 degradation requires activation by light; (ii) neither acceptor nor donor side photoinhibition of PSII induces formation of D1\* *in vitro*; (iii) in isolated thylakoids, the transformation of D1 to D1\* can be induced in low light in the presence of ATP, which suggests that D1\* is a phosphorylated form of the D1 protein; (iv) D1\*, induced either *in vivo* or *in vitro*, is much less susceptible to degradation during illumination of isolated thylakoids than the original D1 protein. We suggest that the modification to D1\* is a means to prevent disassembly of photodamaged photosystem II complex in appressed membranes.

D1 protein; Phosphorylation; Photoinhibition, Photosystem II

### 1. INTRODUCTION

Exposure of intact leaves or different PSII preparations to strong light results in photoinhibition of photosystem II electron transport. The exact mechanism of photoinhibition is still under discussion. When the oxygen evolving side of PSII functions properly, the site of inhibition has been localized to the acceptor side of photosystem II [1–3] as a double reduction of the primary quinone acceptor  $Q_A$  [4] which subsequently leaves its site in the D2 protein [5]. If lesions have been introduced on the donor side of PSII prior to the illumination, photosystem II becomes extremely sensitive to light [6,7], probably due to accumulation of long-lived, highly oxidizing radicals such as  $P680^+$  and tyrosine  $Z^+$  [6–8]. Also, impaired charge separation has been suggested to be the primary event in photoinhibition [9].

Both acceptor and donor side photoinhibition are supposed to lead to an irreversible modification of the D1 protein. In acceptor side photoinhibition, toxic oxygen species are probably involved in the modification [5,10]. In donor side photoinhibition, however, D1 degrades during illumination even in the absence of oxygen [11], and the formation of highly oxidizing species like tyrosine  $Z^+$  or  $P680^+$  have been suggested to modify the structure of the D1 protein [8]. The initial

structural modification(s) of the D1 protein are supposed to expose the cleavage site in the D1 protein and thereby make it susceptible to proteolytic degradation. The protease is probably an integral part of the PSII complex itself [12–14].

*In vivo* photoinhibition experiments with *Chlamydomonas reinhardtii* clearly refer to an irreversible modification of the D1 protein before final degradation [15,16]. Our recent *in vivo* experiments on pumpkin leaves revealed that photoinhibition of PSII electron transport indeed leads to formation of a modified form of the D1 protein, designated D1\* [17]. D1\* migrates in SDS-PAGE slightly more slowly than the original D1 protein. The identity of D1\* has not been resolved, but it seems that D1 degradation *in vivo* occurs via this form. In the present paper, we further study the identity and role of D1\* in the degradation of the D1 protein and indicate that although D1 degradation *in vivo* occurs via D1\*, the latter is in fact a poor substrate for the protease. We also give evidence suggesting that D1\* is a phosphorylated form of the D1 protein.

### 2. MATERIALS AND METHODS

Pumpkin plants were grown in a growth chamber at a photosynthetic photon flux density (PPFD) of  $1000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (12 h) and 23/18°C day/night temperature for 3 weeks.

Thylakoids were isolated [18] from nearly fully expanded leaves after 11 h dark incubation or after 4 h in the light, either at 200 or at  $1000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Tris-washed thylakoid membranes were prepared according to [8].

Isolated thylakoids were suspended in 50 mM phosphate buffer, pH 7.4, containing 0.1 M sucrose, 5 mM  $\text{MgCl}_2$  and 20 mM NaCl. To phosphorylate the thylakoid polypeptides, 0.4 mM ATP was added to the thylakoid suspension and the thylakoids were illuminated at 100

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*Abbreviations:* D1\*, modified form of the D1 protein;  $P680$ , primary donor of photosystem II; PPFD, photosynthetic photon flux density; PSII, photosystem II.

$\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for 20 min. Photoinhibitory illumination was given in a temperature controlled vessel at a PPFD of  $1500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ,  $20^\circ\text{C}$ . A slide projector served as a light source. Chlorophyll concentration during polypeptide phosphorylation and photoinhibitory illumination was  $0.1 \text{ mg/ml}$ .

PSII electron transfer activity from water to phenyl-*p*-benzoquinone ( $1 \text{ mM}$ ) was measured with a Hansatech oxygen electrode at saturating red light as in [18].

Thylakoid polypeptides were separated with SDS-PAGE using a 12–22.5% gradient of acrylamide and  $4 \text{ M}$  urea in the separating gel. The polypeptides were transferred to Immobilon-Lite membrane and the D1 protein was immunodetected by using the chemiluminescence kit of Bio-Rad. The immunoblots were scanned with a laser densitometer.

### 3. RESULTS

#### 3.1. PSII photoinhibition and D1 protein degradation in thylakoids isolated from dark and light acclimated leaves

When thylakoids isolated from dark incubated ( $11 \text{ h}$ ) leaves were subjected to strong illumination, severe photoinhibition of PSII electron transport activity occurred in the course of illumination. Degradation of the D1 protein, however, followed the photoinhibition of PSII extremely slowly (Fig. 1). When similar experiments were performed on thylakoids isolated from leaves illuminated at low light ( $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ,  $4 \text{ h}$ ), oxygen evolution was inhibited with the same kinetics, but now also the D1 protein degraded faster and the degradation more closely followed the inhibition of PSII electron transport. (Only traces of D1\* were induced by the low light incubation of the leaves, see below).

#### 3.2. Amount of D1\* in intact leaves depends on light intensity

When D1 protein was analysed from leaves incubated for  $11 \text{ h}$  in darkness, only a single band was present in

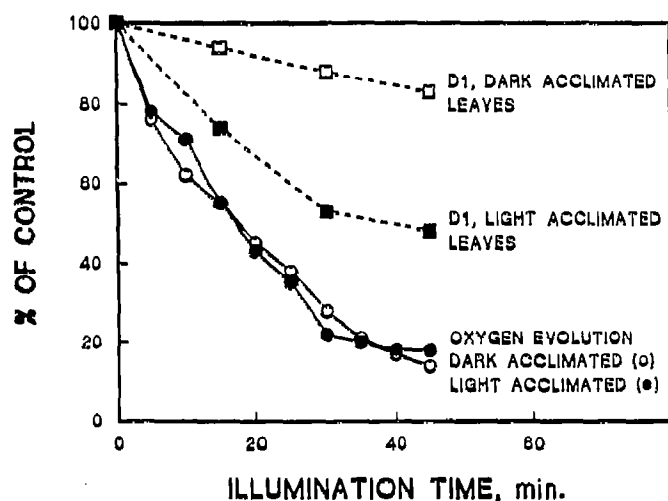


Fig. 1. Photoinhibition of PSII electron transport (○,●) and degradation of the D1 protein (□,■) during illumination of thylakoid membranes isolated from leaves after long, dark incubation ( $11 \text{ h}$ ) (open symbols) or after incubation in light ( $200 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) for  $4 \text{ h}$  (closed symbols).

the immunoblots (Fig. 2, lane 1). In addition to this original D1, a novel form of the D1 protein, D1\*, was evident if the leaves had been illuminated prior to thylakoid isolation (Fig. 2, lanes 2–4). D1\* migrates in SDS-urea-PAGE slightly more slowly than the original D1. The proportion of D1\* from total D1 content depended on the photon flux density during light acclimation of the leaves. Only traces of D1\* could be detected in leaves illuminated at a low PPFD of  $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , and with increasing light level, more of the D1 protein was present as D1\*. However, even in the photoinhibitory PPFD of  $3000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (which induces 70% inhibition of PSII electron transport in  $3 \text{ h}$ ), D1\* did not exceed 50% of the total D1 protein content of the thylakoid membranes. Also, no net loss of the D1 protein (D1 + D1\*) occurred in spite of severe photoinhibition of PSII in intact leaves as also reported earlier [17].

#### 3.3. Formation of D1\* in isolated thylakoids

Because the presence of D1\* in intact leaves is light-dependent, we tested if illumination of isolated thylakoids also induces D1\*, possibly as a consequence of photoinhibition. In order to test if D1\* is produced by irreversible modification of the D1 protein [19] in ac-

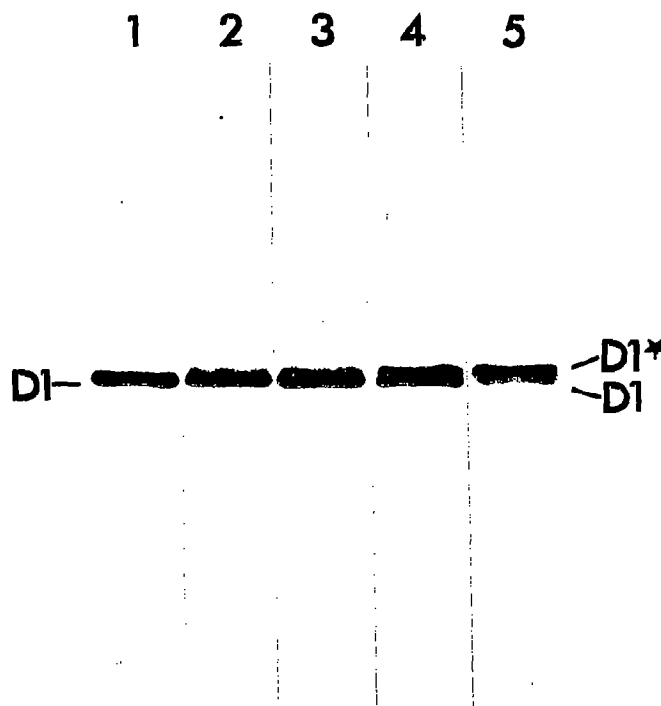


Fig. 2. Immunoblot of the D1 protein demonstrating the light-dependent appearance of the modified form of the D1 protein, D1\*. (Lane 1) thylakoids isolated from dark-acclimated leaves ( $11 \text{ h}$ ); (lane 2) leaves acclimated to  $200 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for  $4 \text{ h}$ ; (lane 3) to  $1000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for  $4 \text{ h}$ ; and (lane 4) to  $3000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for  $3 \text{ h}$ . Light treatment at  $3000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for  $3 \text{ h}$  induced 70% inhibition of PSII electron transport. (Lane 5) as in lane 2 but the isolated thylakoids were incubated in low light in the presence of ATP (see Materials and Methods).  $2 \mu\text{g}$  chlorophyll was applied to each well.

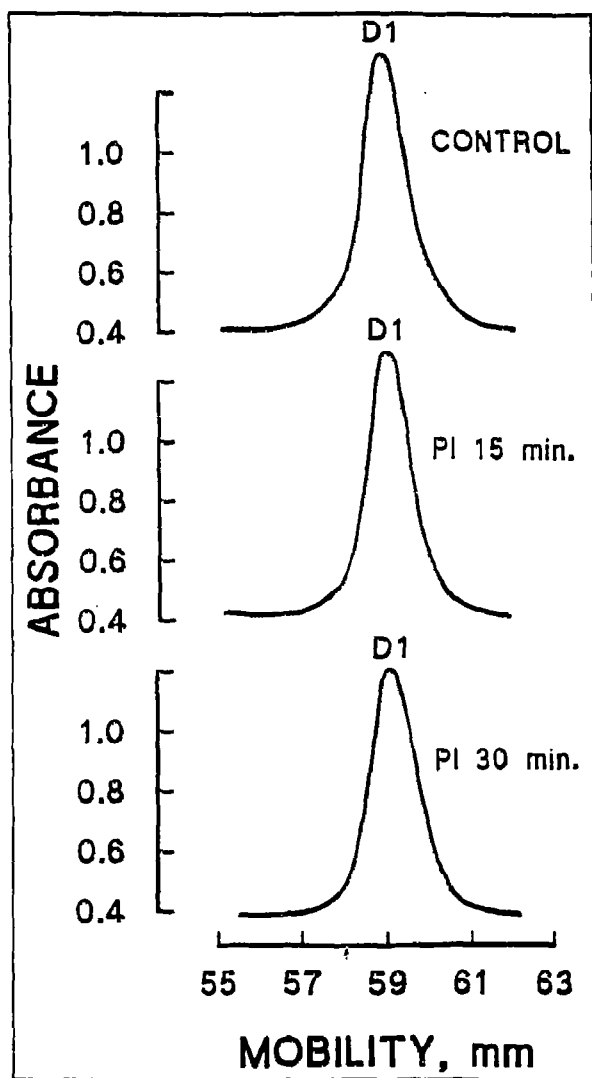


Fig. 3. Laser densitograms of immunoblots of the D1 protein demonstrating that D1 is not transformed to D1\* in the course of photoinhibition of PSII activity in vitro. Thylakoids were isolated from dark-incubated (11 h) leaves in order to minimize D1 degradation in the course of photoinhibition. Illumination was performed in the absence of electron acceptors. Illumination ( $1500 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ) of 15 and 30 min induced 45 and 75% inhibition of PSII activity, respectively. PI = photoinhibitory illumination.

ceptor side photoinhibition, thylakoids were illuminated without added external electron acceptors. In the in vitro experiment, photoinhibition of PSII electron transfer was not accompanied by formation of D1\* (Fig. 3). Dark-acclimated leaves were used in order to avoid simultaneous D1 degradation, but neither could we see any increase in D1\* during photoinhibition of thylakoids of light-acclimated leaves (Fig. 4A). Also, when the thylakoids were Tris-washed to inactivate the oxygen evolving complex, and subsequently photoinhibited, no formation of D1\* could be detected (results not shown).

Apart from photoinhibition, we were able to induce

a transformation of D1 to D1\* in isolated thylakoids under phosphorylating conditions (Fig. 2, lane 5). When thylakoids isolated from dark- or light-acclimated leaves were illuminated ( $100 \mu\text{mol} \cdot \text{photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for 20 min) in the presence of 0.4 mM ATP, the D1 protein was almost completely transformed to D1\*. This transformation did not affect the light-saturated PSII activity; 20 min illumination of thylakoid membranes both in the presence and absence of ATP similarly decreased the light-saturated PSII activity by 10% from the control value of  $204 \mu\text{mol} \cdot \text{O}_2 \cdot (\text{mg Chl})^{-1} \cdot \text{h}^{-1}$ . The D1\* formed in isolated thylakoids in the presence of ATP was very stable and transformation back to D1 was observed neither in darkness nor in the light during subsequent experiments. Only incubation of thylakoids with alkaline phosphatase [20] partially transformed D1\* back to D1 (data not shown). These experiments suggest that D1\* is a phosphorylated form of the D1 protein. Further support for this interpretation comes from experiments with membranes from a cyanobacterium, whose D1 protein cannot be phosphorylated [21]. D1\* was not formed in the membranes of *Synechocystis* 6803 in our phosphorylating conditions (data not shown).

#### 3.4. D1\* is protected against degradation

The formation of D1\* does not seem to be an immediate consequence of photoinhibition of PSII electron transport, and D1\* as such does not seem to represent a light-induced, irreversibly modified form of the D1 protein that is supposed to be degraded. To study the nature of D1\*, we tested which form of the D1 protein is more readily degraded during photoinhibition of isolated thylakoids. Thylakoids isolated from light-acclimated leaves were illuminated at  $100 \mu\text{mol} \cdot \text{photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for 20 min in the presence of ATP to transform most of the D1 protein to D1\* (Fig. 2, lane 5). Subsequent illumination of both ATP-treated and control thylakoids at  $1500 \mu\text{mol} \cdot \text{photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  resulted in similar kinetics of inhibition of oxygen evolution as in Fig. 1. Significantly, D1\* was clearly protected against degradation in both kinds of thylakoids (Fig. 4), and the original D1 protein was the one that was degraded during illumination. Interestingly, the in vivo-produced D1\* that was present in thylakoids isolated from light-acclimated leaves (Fig. 4A) and the in vitro-induced D1\* (Fig. 4B) were similarly protected against degradation.

#### 4. DISCUSSION

The rate of the D1 protein turnover in the thylakoid membrane is light-dependent [22–25]. It has been postulated that during strong illumination, concomitantly with photoinhibition of PSII activity, the D1 protein becomes covalently modified as a consequence of radical or singlet oxygen attack [26]. It has been argued that

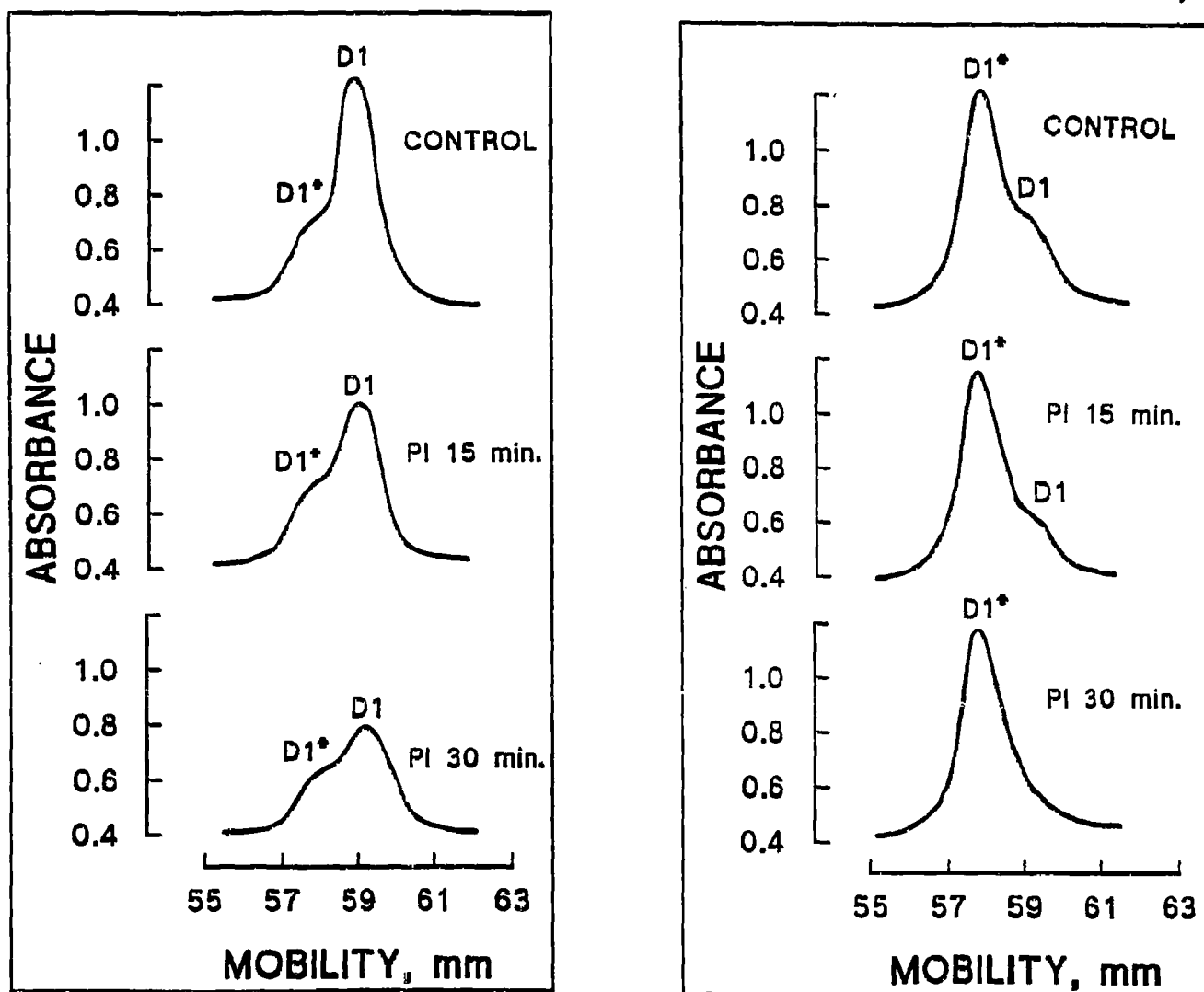


Fig. 4. Laser densitograms of immunoblots of the D1 protein demonstrating degradation of D1 but not D1\* during illumination of thylakoid membranes. Thylakoids were isolated from leaves previously acclimated to  $1000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for 4 h to ensure that the protease was active and that D1\* was present. (A) control thylakoids; (B) same thylakoids as in A but treated with ATP prior to photoinhibitory illumination (PI). Photoinhibition of PSII activity followed the same kinetics as in Fig. 1.

it is only after this modification that the cleavage site of the D1 protein becomes properly exposed for a specific protease. However, our present results strongly suggest that it is not only the D1 protein that has to be activated via photoinhibition of PSII but probably also the protease itself requires light activation. Degradation of the D1 protein is severely inhibited during strong illumination of thylakoids isolated from dark-acclimated leaves (Fig. 1). Still, photoinhibition of PSII electron transport and thereby most probably also the covalent modification of the D1 protein proceed with the same rate in thylakoids isolated from dark-acclimated leaves and from leaves acclimated to low light. The protease cannot be activated by illuminating isolated thylakoids as such, but obviously reactions requiring additional cofactors from stroma (e.g. ATP) are involved. Many of the proteolytic systems in chloroplasts are known to

be ATP-stimulated [27,28]. The exact nature of the light activation of the D1-specific protease remains to be elucidated.

During *in vivo* photoinhibition of pumpkin leaves the D1 protein is degraded via a modified form, designated D1\* [17]. Similar modification of the D1 protein (32\*) has earlier been reported in *Spirodela* cells during normal D1 turnover in low-light conditions [29]. Whether these novel forms of the D1 protein represent the so-called triggered form of the D1 protein which is an actual substrate for proteolytic degradation could, however, not be directly answered on the basis of *in vivo* experiments. Therefore a set of *in vitro* experiments were designed to elucidate the origin and role of D1\*. Neither photoinhibition of isolated thylakoids in the absence or presence of external electron acceptors nor photoinhibition of thylakoid membranes with non-

functional oxygen-evolving complex induced formation of D1\*. Still, both these photoinhibition designs are known to mark the D1 protein for degradation [8,19]. These results strongly suggest that D1\* as such is not the covalently modified form of the D1 protein that is argued to be the proper substrate for the protease. Further evidence for this interpretation comes from the fact that D1\* can be induced in isolated thylakoids without photoinhibition of PSII electron transport. Treatment of thylakoid membranes in phosphorylating conditions (ATP and low light) induces D1\* without inducing photoinhibition. As D1 is known to be a phosphoprotein [30], we suggest that D1\* represents a phosphorylated form of the D1 protein. Slightly slower migration in SDS-urea-PAGE gels is also seen after phosphorylation of other PSII polypeptides [31].

To find out which form of D1 is degraded, we isolated thylakoids from light-acclimated leaves to ensure that the protease was active. Treatment of these thylakoids with ATP induced nearly complete transformation of D1 to D1\*. To our surprise, the subsequent photoinhibition experiments with ATP-treated and non-treated thylakoids clearly demonstrated that D1\* is actually protected from proteolysis. In vitro-produced D1\*, and the D1\* that was produced in vivo in a leaf, behaved similarly during in vitro photoinhibition of isolated thylakoids.

We have earlier shown that degradation of the D1 protein occurs via this modified form in intact leaves [17]. In low-light-grown plants with relatively slow turnover of the D1 protein, nearly complete transformation of D1 to D1\* precedes D1 degradation. An interesting hypothesis is that D1 is modified (phosphorylated?) in photodamaged PSII centers to retard its degradation. D1\* is present only in the appressed thylakoid membranes [17,29] while new D1 is inserted to stroma thylakoids [32]. Possibly the modification of the D1 protein is a means to prevent total disassembly of photodamaged PSII complexes in grana membranes and to ensure synchronization of degradation and synthesis of D1 in stroma thylakoids. Also, for low-light-grown plants, it would not indeed be advantageous to spend scarce energy resources for fast D1 turnover. Retardation of D1 degradation in appressed membranes might also ensure excitation energy quenching [9] in these centers and thereby protect the photosynthetic apparatus from totally irreversible photo-oxidative damage. How the D1 protein then finally is degraded, whether transformation of D1\* to D1 is a prerequisite for degradation and whether stroma localized phosphatases are involved in this process, are questions which are presently under study.

*Acknowledgements:* This work was supported by the Academy of Finland. Prof. I. Ohad is gratefully acknowledged for the D1 antibody. The authors thank Prof. B. Andersson for stimulating discussions.

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