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D1-D2 protein degradation in the chloroplast
Complex light saturation kinetics

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The D1 and D2 proteins of the photosystem II (PSII) reaction center are stable in the dark, while rapid degradation occurs in the light. Thus far, a quantitative correlation between degradation and photon fluences has not been determined. In *Spirodela oligorrhiza*, D1-D2 degradation increases with photon flux. We find that kinetics for D2 degradation mirror those for D1, except that the actual half-life times of the D2 protein are about three times larger than those of the D1. The degradation ratio, D2/D1, is fluence independent, supporting the proposal [Jansen, M.A.K., Greenberg, B.M., Edelman, M., Mattoo, A.K. & Gaba, V. (1996), *Photochem. Photobiol.* **63**, 814–817] that degradation of the two proteins is coupled. It is commonly conceived that D1 degradation is predominantly associated with photon fluences that are supersaturating for photosynthesis. We now show that a fluence as low as 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ elicited a reaction constituting $> 25\%$ of the total degradation response, while $> 90\%$ of the degradation potential was attained at intensities below saturation for photosynthesis (≈ 750 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Thus, in intact plants, D1 degradation is overwhelmingly associated with fluences limiting for photosynthesis. D1 degradation increases with photon flux in a complex, multiphasic manner. Four phases were uncovered over the fluence range from 0–1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The multiphasic saturation kinetics underscore that the D1 and D2 degradation response is complex, and emanates from more than one parameter. The physiological processes associated with each phase remain to be determined.

Keywords: chloroplasts, *Spirodela oligorrhiza*, photosystem II, protein degradation.

Photosystem II (PSII) is a highly structured pigment-protein complex that catalyses the primary photochemistry leading to oxygen evolution and electron flow in oxygenic phototrophs [1,2]. The PSII reaction center is dominated by the D1/D2 heterodimer core [1,2]. A characteristic feature of this core is the rapid, photon-flux dependent turnover of the D1 protein [3–5] and, under certain conditions, the D2 protein as well [6–9]. At least two different photosensitizers are involved in the mediation of D1 degradation [10] with interactions between the two resulting in synergistic enhancement of the process [11]. Degradation of D1 and D2 is driven by a range of wavelengths broader than for PSII activity, the biologically relevant spectrum extending from UV-B, through UV-A, photosynthetically active radiation (PAR) and into the far red [9,10,12].

The D1 and D2 proteins undergo a number of well-studied post-translational modifications during their life cycles. These include: additions of chlorophyll, pheophytin, quinone, carotenoid, bicarbonate and a nonheme iron molecule [1]; radicalization of chlorophylls, tyrosines and quinones [13]; acetylation [14], palmitoylation [15] and reversible phosphorylation of N-terminal threonines [14,16,17]. Similarly, a number of metabolic life-cycle events has been documented, such as: ribosomal pausing [18], N-terminal [14] and C-terminal processing [19–21], internal crosslinking [22], aggregation [13] and reducing-[23,24] and oxidizing-side [25,26] degradation. In addition, there are specific interactions of the D1/D2 heterodimer with the water-splitting and accessory-pigment protein complexes of PSII [1]. Hypotheses, some mutually exclusive, concerning the effects of some of these modifications on D1 metabolism are abundant [13,17,27–29].

The D1/D2 heterodimer is an integral membrane complex, its proteinaceous components readily solubilized only by ionic detergents. This fact has limited methods of analysis. Two main approaches have been taken to study D1 turnover in the intact plant: one is the measurement of PSII functionality as an indirect assessment of D1 turnover [30]; however, more frequently, radio-pulse-chase experiments [31] or immunological techniques [6,32] have been employed to follow the appearance or disappearance of the D1 and D2 protein bands on detergent-containing, denaturing gel matrices (e.g. SDS/PAGE). A number of these latter studies have specifically confirmed aggregates [13], conformers [33] or break-down products [23,25,26] of D1 or D2. In this work, we have used a radio-pulse-chase technique to study *in vivo* the time-dependent disappearance of radiolabel from the mature D1 or D2 gel band, a process we shall refer to as degradation.

In the intact plant, D1 and D2 degradation has been demonstrated to occur under photon fluences that are rate limiting [3,9,10,32,34] or supersaturating (i.e. photoinhibitory) [6,17,34–40] for photosynthesis. Many theories [13,17,27,29] address the relationship between D1 degradation and photoinhibition, and a few between D1 degradation and low intensity light [3,12,32]. No theories exist for mechanisms that control D2 instability. For the degradation of either protein there is a lack of data on photon-fluence saturation kinetics.

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Abbreviations: PSII, photosystem II; PAR, photosynthetically active radiation.

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The acquisition of fundamental insight in the turnover of PSII requires the dissection of the complex mixture of assembly and disassembly processes in relatively simple reactions. In this study, we have determined and quantified the saturation kinetics of the photon-flux dependency of the degradation of the D1 and D2 proteins, using a pulse-chase technique. We demonstrate that in the intact plant, D1 and D2 degradation are processes overwhelmingly associated with fluences limiting for photosynthesis. The kinetics are shown to be complex, with distinct multiphasic jumps and plateaus at specific fluences, but the ratio of D1 vs. D2 degradation is fluence independent.

EXPERIMENTAL PROCEDURES

Plant material

Axenic cultures of *Spirodela oligorrhiza* (*S. punctata*) were grown phototrophically on half-strength Hutner's medium [41] in a CO_2-enriched atmosphere (1–3%) under continuous irradiation. The fluence rate during growth was 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR (cool-white fluorescent bulbs), unless otherwise indicated.

Assay for D1 and D2 protein degradation

Degradation of the D2 and D1 proteins was measured *in vivo* by pulse-chase experiments [10]. *Spirodela* fronds were pulse-labeled with [^{35}S] methionine for 2 h under 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of PAR, rinsed and chased for various periods of time in growth medium containing 1 mM nonradioactive methionine. Radiation conditions are detailed in the figure legends. The chase period depended upon the fluence to which plants were exposed; at higher fluence rates, the chase period was shorter. For example, time points were taken at 4, 8, and 20 h for 6 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, and at 2, 4, and 8 h for 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$. All calculations of kinetics were based on data points taken within the first two half lives of the proteins.

Following the chase, membrane proteins were isolated, fractionated by SDS/PAGE on 10–20% gradient slab gels and visualized by autoradiography [10]. The D1 and D2 proteins have molecular weights of 38 and 39.5 kDa, respectively [1]. Degradation of the proteins (i.e. the rate of disappearance of the pulse-labeled bands on polyacrylamide gels) was quantified by microdensitometry, and the data normalized to those for the stable lightharvesting chlorophyll a/b protein band (26 kDa) [23]. Identity of bands were periodically checked by immunoblotting. The *in vivo* half-life times were determined for each individual sample point by comparison with the 0 h time point, assuming first-order kinetics [10]. The reciprocal of the half-life time (h-1) was used as a measure of the rate constant of protein degradation. A typical autoradiograph, depicting D1 and D2 degradation *in vivo*, in *Spirodela*, is shown in Jansen *et al.* [11].

Reciprocals of the half-life time of the D1 or D2 proteins were plotted vs. photon fluence and a logarithmic curve was fitted. At each photon fluence it was determined whether the measured data deviated significantly (*t*-test) from the best-fitting single logarithmic curve (STATVIEW and STATWORKS).

Radiation sources and photon fluence measurements

Radiation sources were as follows: broad-spectrum visible light was generated by either cool-white fluorescent tubes (up to 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or a 250-W tungsten-halogen projector fitted with three glass lenses, a 2.5-cm-thick water filter and a 2-mm KG3 heat absorbing glass (range from 25 to 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$); 429 and 660 nm wavelengths (Schott, Mainz, Germany, half-power band width 10–20 nm) were generated using a tungsten-halogen projector fitted with a heat absorbing glass plus the appropriate interference filter. Far red was generated by filtering the light of a halogen flood lamp through a 180-mm-thick water filter, a 2-mm KG3 heat absorbing glass and a Wratten 89B filter (Kodak, USA). Photon fluences were adjusted by varying the distance of the sample from the light source. Fluences were measured using a LI-189 quantum sensor (Li-Cor Inc., Lincoln, NE, USA).

Measurements of oxygen evolution

Photoacoustic spectroscopy was used to quantify oxygen evolution in intact *Spirodela* plants [42]. Oxygen signals are proportional to the quantum yield of oxygen evolution [42]. Measuring light (20 $\mu mol \cdot m^{-2} \cdot s^{-1}$) modulated at a frequency of 15 Hz, was directed via optic fibers to a tightly closed cell, containing sample and microphone sensor. Microphone signals were processed by a lock-in amplifier (SR530, Stanford Research Systems, Thousand Oaks, CA, USA) using vectorial analysis. Oxygen evolution was saturated, and the photothermal signal was maximized, by applying 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of nonmodulated PAR [42].

RESULTS

Multiphasic fluence dependency of D1 degradation

We systematically measured the photon-flux dependency of D1 degradation *in vivo*, in *Spirodela*. Plants were grown for several

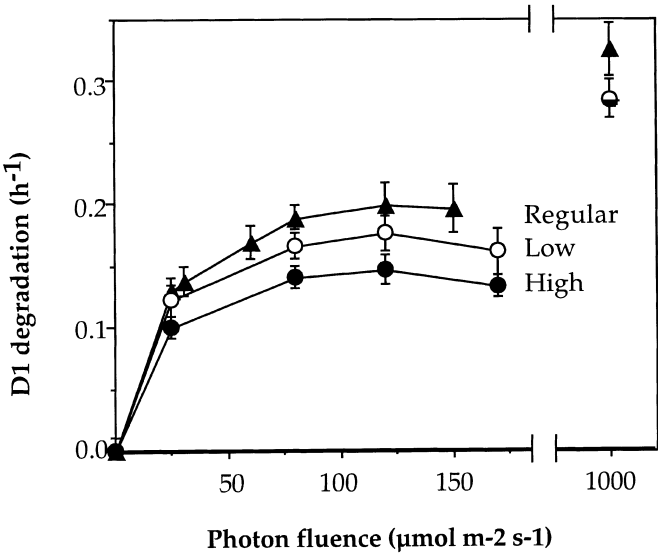

Fig. 1. D1 protein degradation in *Spirodela* plants raised under different photon fluxes. *Spirodela* plants were grown for ≥ 10 generations either in closed Erlenmeyer flasks at a fluence of 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$, or in open Petri dishes at fluences of 6 $\mu mol \cdot m^{-2} \cdot s^{-1}$ or 85 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR (regular, low and high, respectively). Plants were radiolabeled with [^{35}S] methionine for 2 h under 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of PAR, rinsed and chased for various periods of time at the photon fluences indicated, as described in Materials and Methods. Plants were homogenized and the membrane protein fraction isolated and fractionated by SDS/PAGE [10]. Radiolabeled protein bands were detected by autoradiography and their degradation kinetics determined as described [23]. h^{-1} is the reciprocal of the half-life time in hours. Values represent averaged data from several experiments (for each photon fluence point, n = 14–30; total number of gel lanes analyzed = 350). Standard errors of the mean are shown.

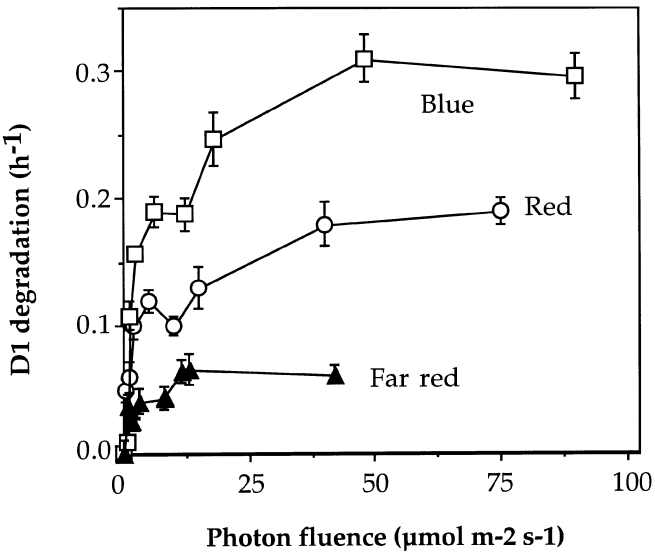

Fig. 2. Degradation of the D1 protein *in vivo* as a function of low photon-flux densities. *Spirodela* oligorrhiza plants were radiolabeled with [^{35}S] methionine for 2 h under 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of PAR, rinsed and chased for various periods of time at the photon fluences indicated, as described in Materials and Methods. The light quality during the chase was either blue (429 nm), red (660 nm) or far red (720 nm) radiation. Protein degradation was determined as described in the legend to Fig. 1. h^{-1} is the reciprocal of the half-life time in hours. Values represent averaged data from several experiments (for each photon fluence point, $n = 18$; total number of gel lanes analyzed = 450). Standard errors of the mean are shown.

generations under either 6, 25 or 85 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR. In all cases, D1 degradation strongly accelerated in response to increasing photon fluences in the range between 0 and 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Surprisingly, however, the fluence dependency curves leveled off between 25 and 170 $\mu mol \cdot m^{-2} \cdot s^{-1}$, supporting the impression that saturation had been reached (Fig. 1). We analyzed this phenomenon in more detail by increasing the density of experimental points and separately examining the effects of the main qualitative components of PAR on the system. The results, summarized in Fig. 2, show that blue, red and far-red radiation each support rapid acceleration of D1 degradation at low fluences, however, the slopes of acceleration evidently leveled off at fluences higher than 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$, creating an impression that the process had reached saturation (Fig. 2). Moreover, in the fluence range of 3–9 $\mu mol \cdot m^{-2} \cdot s^{-1}$, there were clear indications for deviation from a single logarithmic curve at each wavelength tested (Fig. 2).

The fluence range studied was extended from the low fluences to the equivalent of 12.00 h terrestrial sunlight (1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Fluences greater than 170 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, triggered a further enhancement of D1 protein degradation in all *Spirodela* cultures tested (Fig. 1, 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ point; Fig. 3). Detailed analysis of the full range up to 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ showed a conspicuous additional deviation from a single logarithmic curve in the range 400–650 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Fig. 3). Further increases in the fluence from 650 to 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR did not significantly enhance degradation.

D2 protein degradation mirrors that of D1

Measurements of D2 degradation were made in parallel to those of D1. The photon-flux dependency curve for D2 protein degradation in *Spirodela* shows that the D2 protein, like D1, is catabolized under PAR, however, the rate constant is about threefold smaller. The fluence dependency of D2 degradation strikingly mirrors that of D1 (Fig. 3). As a result, the ratio of their rate constants of degradation (D2/D1) is fluence independent over the range studied. Similarly, when blue or red radiation-driven degradation was determined, it was found that the ratio (D2/D1) of the rate constants of degradation was fluence independent over the range from 1 to 75 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (not shown).

The photon-flux dependency curves for D1 and D2 degradation (Fig. 3) are each derived from 20 fluence points experimentally repeated, on average 20 times. This mass of data allowed statistical analysis of the fluence dependence of D1 and D2 degradation. A *t*-test revealed highly significant (≈99%) deviations from a monophasic logarithmic saturation curve in the fluence ranges of 80–250 and 400–650 $\mu mol \cdot m^{-2} \cdot s^{-1}$. A further deviation (*t*-test ≈99%) from a single logarithmic curve occurred at fluences of 3–9 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at all four wavelengths studied (Fig. 2,3). Thus, *in vivo*, in *Spirodela*, over the physiological fluence range of 1–1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$, the kinetics of degradation for both the D1 and D2 proteins are multiphasic.

Oxygen evolution and photoinhibition

The fluence dependence of D1 degradation *in vivo* (Fig. 3) was compared with the saturation kinetics of oxygen evolution and

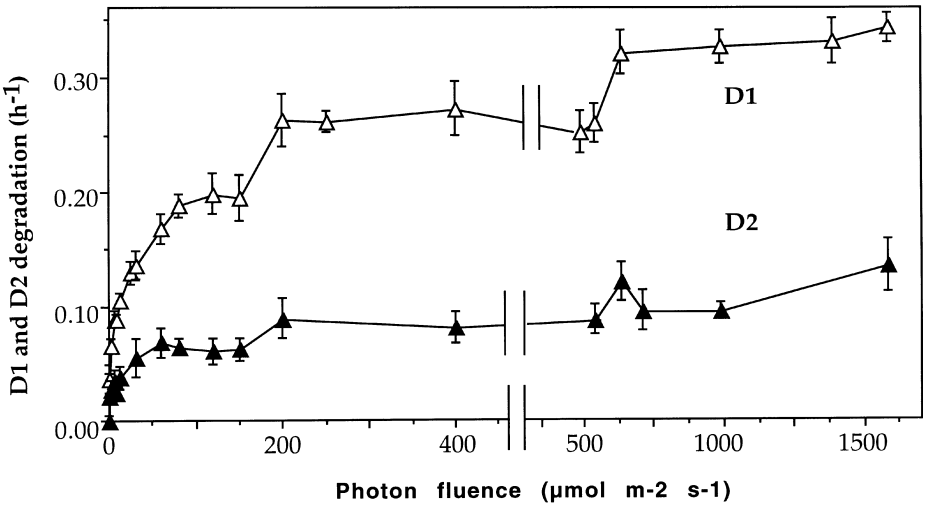

Fig. 3. Photon-flux dependency of D1 and D2 degradation in *Spirodela* over the range from 0 to 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Plants were radiolabeled, chased at the photon fluences indicated, and protein degradation determined as described in the legend to Fig. 1. Measurements of the D1 and D2 protein bands were take in parallel for all gel lanes. h^{-1} is the reciprocal of the half-life time in hours. Values represent averaged data from several experiments (for each photon fluence point, $n_{D1 \pm D2} = 14–30$; total number of gel lanes analyzed = 400). Standard errors of the mean are shown.

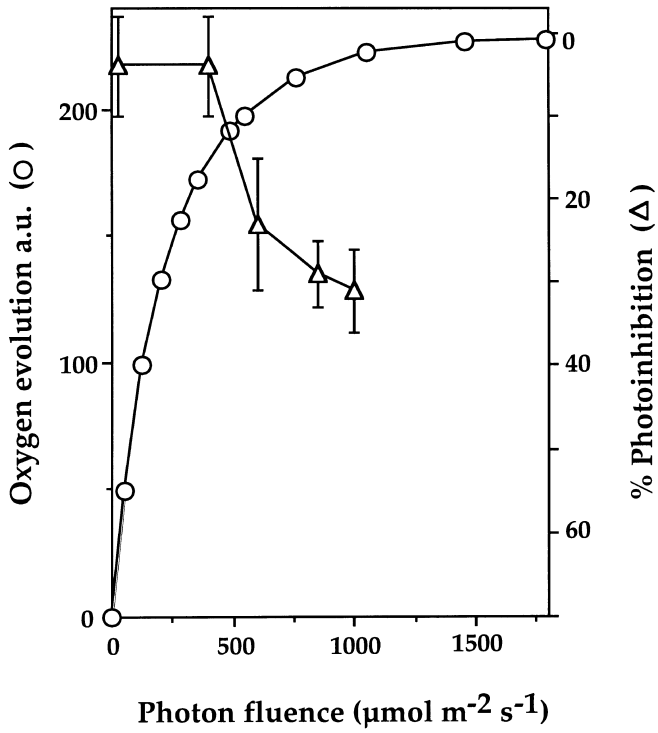

Fig. 4. Relative rate of oxygen evolution as a function of photon flux in *Spirodela*. Quantum yields of oxygen evolution were measured *in vivo* under a range of fluences by photoacoustic spectroscopy [11]. A saturation curve was constructed by integrating the flux dependency of the quantum yield of photosynthetic oxygen evolution. Photoinhibition was measured as a decrease in the quantum yield of oxygen evolution after exposure for 3 h to visible light at the fluence indicated. The quantum yield that was measured prior to the exposure was taken as 100%. Values represent averaged data from several experiments. For each curve at each photon fluence point $n = 6$. Standard errors of the mean are shown for photoinhibition.

photoinhibition *in vivo*, each parameter representing an aspect of the functionality of the photosynthetic light reactions. Relative rates of oxygen evolution, measured by photoacoustic spectroscopy, were ≥ 95% saturated at a fluence of 750 μmol·m^{-2}·s^{-1} (Fig. 4). The relative differential quantum yield of oxygen evolution decreased (i.e. photoinhibition) after prolonged exposure (3 h) to fluences of 600 μmol·m^{-2}·s^{-1} PAR or more (Fig. 4). Exposure to these fluences yielded comparable decreases in relative variable fluorescence (data not shown).

DISCUSSION

D1 degradation is a low-fluence event in the intact plant

A key step in the turnover of the PSII reaction centre protein D1, is its light dependent degradation. We found that a fluence as low as 5 μmol·m^{-2}·s^{-1} elicited a reaction constituting ≥ 25% of the total degradation response at 1600 μmol·m^{-2}·s^{-1}, while a fluence of 200 μmol·m^{-2}·s^{-1} triggered ≥ 75% of the response (Fig. 5). Thus, D1 degradation is a process essentially associated with low fluences in the intact plant. Agreement is emerging on this point. Using pulse-chase methodology, it was demonstrated that in *Brassica* leaves D1 degradation is mainly associated with low (≤ 350 μmol·m^{-2}·s^{-1}) photon fluences [34]. Likewise, using immunodetection, D1 degradation in chloramphenicol-treated *Chlamydomonas* cells was demonstrated to be largely associated with relatively low photon fluences [32].

In *Spirodela*, saturation of D1 and D2 degradation (Fig. 3) roughly coincides with the saturation of photosynthetic oxygen evolution (Fig. 4). Thus, in the intact plant, protein degradation is overwhelmingly associated with fluences limiting for oxygen evolution. This does not necessarily mean that the two processes are coupled. Previously, we have shown that far red and UV radiation drive D1 degradation efficiently, despite the lack of significant linear PSII electron flow under these wavelengths [11,12]. We have also shown that the PSII inhibitor BNT (unlike some of its sidechain substituted analogues) does not affect D1 degradation at concentrations that abolish linear PSII electron flow [28]. However, while linear electron flow through PSII can be uncoupled from D1 and D2 degradation, it is still possible that other PSII electron transfer events are directly linked to degradation [43].

The association of D1 and D2 degradation with fluences that are limiting for photosynthesis is further underlined by the observation that photoinhibition becomes apparent only at photon fluences ≈600 μmol·m^{-2}·s^{-1} or greater (Fig. 4). Thus, saturation of D1/D2 degradation coincides with the onset of detectable photoinhibition (Figs 2 and 4).

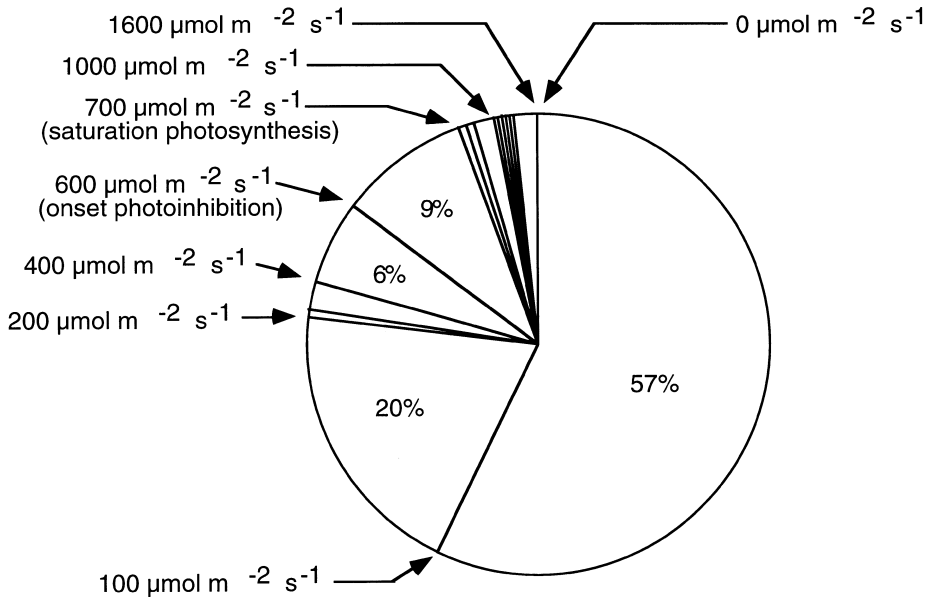

Fig. 5. The relative contribution of fluence ranges to D1 degradation. *Spirodela* plants were radiolabeled, chased in the light, and protein degradation was determined as described in the legend to Fig. 1. The area of the pie chart represents the total degradation response of the D1 protein, as measured at 1600 μmol·m^{-2}·s^{-1}. The pie is divided in segments, each of which represents a fluence range of 100 μmol·m^{-2}·s^{-1}. Several key fluences are indicated adjacent to the pie chart. The size of each segment reflects the percentage of the degradation response which is contributed by a given photon fluence range. Values represent averaged data from several experiments, as detailed in legends to Fig. 3.

D2 protein degradation is coupled to that of the D1 protein

The photon-flux saturation curve for D2 protein degradation resembles that for D1, except that half-life times of the D2 protein are about 3 times larger than those of D1 (Fig. 3). Thus, throughout the range measured, the degradation ratio, D2 vs. D1, is largely fluence independent, suggesting that degradation of the two proteins is coupled. Several lines of evidence strengthen this observation: (a) the *in-vivo* D2 degradation spectrum strongly resembles that for D1 [9]; (b) PSII inhibitors have parallel effects on D1 and D2 degradation [9]; (c) in isolated PSII reaction centers, both D2 and D1 degradation involve two distinct pathways attributed to donor-and acceptor-side damage [44]; (d) absence of one PSII reaction center protein in deletion mutants results in destabilization of the other [45]; (e) site-directed mutagenesis in the *D-de* loop region of D2 affects the QB-binding environment [46] which is created exclusively by residues of the D1 protein [47]. Likewise, in isolated thylakoids, trypsinization of both D2 and D1 is influenced by PSII inhibitors interacting with the QB-pocket. Although D2 is the primary trypsin target, its availability is apparently controlled through the D1 protein [48]. At present, we do not know the mechanism that underlies the coupled degradation of the two proteins. Recently, it was hypothesised, based on *in vitro* experiments, that the D2 protein acts as a receptor component for newly synthesised D1 protein [39]. Our data show an appreciable rate of D2 degradation indicating that, in one out of three D1-turnover events, reaction centre re-assembly must follow a different route. Such an additional mechanism will be especially important under conditions of UVB radiation exposure, where we found that the rate of D2 degradation may approach that of the D1 protein [11].

PSII reaction center protein degradation occurs in multiphasic steps *in vivo*

The photon fluence response curve for D1 protein degradation in intact *Spirodela* plants is unexpectedly complex. Four distinct phases were uncovered over the fluence range from 0 to 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Fig. 3). Highly significant ($\geq$ 99%, *t*-test) deviations from monophasic logarithmic saturation kinetics were found in the range of each rise. Phase I, representing $\approx$25% of the total degradation capacity, extended from 0 to 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Phase II, representing $\approx$30% of the total degradation capacity, extended from 10 to 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Phase III, representing $\approx$20% of the total degradation capacity, extended from 150 to 550 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Finally, phase IV, representing $\approx$25% of the total degradation capacity, extended from 550 to 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

How universal are these phases? We can also discern a multiphasic trend in the photon-flux dependency data of Keren *et al*. [32] for D1 degradation in *Chlamydomonas* which parallels the situation described here for *Spirodela*. A complex photon fluence dependence indicates that different parameters determine the rate constant of degradation at different fluences. In line with this, it was found that some site-specific D1 mutations accelerate degradation under low photon fluences but not under saturating ones [49]. Such effects are not yet understood. However, the detailed description of the saturation kinetics for D1/D2 degradation (Fig. 3) will facilitate comparisons of low-light and high-light type degradation. Using low photon fluences, Ohad *et al*. [32] discovered a tight correlation between degradation of the D1 protein *in vivo* and the recombination between Q_B^- or Q_A^- and oxidized S2 or S3 [43]. Our data emphasise that the mechanisms underlying low light D1-D2 degradation are of prime importance, as they drive more than half of the total degradation response. At the opposite end of the fluence scale photoinhibitory PSII reaction center degradation would probably be limited to phase IV. This might include donor or acceptor side inactivation which are thought to occur at such fluences [27]. Alternatively, it can not be excluded that the multiphasic kinetics (Fig. 3) reflect the existence of distinct PSII populations characterised by differences in functional stability [37]. The D1/D2 heterodimer proteins undergo quite a number of modifications during their life cycle. For example, differentially phosphorylated PSII populations have been isolated and, interestingly, these were found to differ in their sensitivity to strong light [50]. In summary, the multiphasic saturation kinetics underscore the fact that the D1 and D2 degradation response is complex, and emanates from more than one single parameter. The physiological roles of the D1 and D2 degradation phases distinguished here remain to be identified.

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