Degradation of the 32 kD Herbicide Binding Protein in Far Red Light[1]

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ABSTRACT

White light (400–700 nanometers) supports the activity of photosystem I (PSI) and photosystem II while far red light (≥700 nanometers) supports PSI almost exclusively. In intact fronds of *Spirodela oligorrhiza*, turnover of the 32 kilodaltons herbicide binding protein is stimulated under both these light conditions, although not in the dark or at wavelengths >730 nanometers. As is the case in white light, the far red light induced degradation of the protein is inhibited by DCMU. The means by which far red light operates is unclear. Hypotheses considered include: PSI activated proteolysis, PSI-induced formation of semiquinone anions, and PSI-generated free radicals.

The rapidly degraded 32 kD thylakoid protein has been postulated to function as a regulatory component of PSII (19). It is an integral part of the PSII core complex (20) and, most likely, a reaction center protein (3, 30). The 32 kD protein binds quinones (31) and is the site of action of triazine and diphenyl urea herbicides (10).

In a previous study (17) we showed that the rate of turnover of the 32 kD protein is strongly influenced by light intensity (fluence rate). Degradation of this protein was linked to photosynthetic electron transport, this process being effectively blocked by PSII inhibitors such as DCMU and atrazine. In line with these findings, we considered it likely that PSII activity would be a specific requirement for degradation.

This hypothesis was tested by following degradation of the protein in FR[5] light. PSI and PSII differ in threshold quantum energy requirements. As a result, PSI can be driven by a narrow band of wavelengths in the FR part of the spectrum in which PSII is inactive. Unexpectedly, it was found that FR light promoted turnover of the 32 kD protein and that degradation was inhibited by DCMU. These data and their implications are presented here.

MATERIALS AND METHODS

Radiolabeling Conditions. Experiments were performed using axenic cultures of *Spirodela oligorrhiza* (Kurtz) Hegelm grown autotrophically on half-strength Hutner's medium (23). All dark manipulations were carried out with the aid of a dim green safelight. Synthesis of the 32 kD protein was determined as follows: radiolabeling was started in darkness by the addition of [35S]methionine (>800 Ci/mmol; Amersham) to a concentration of 0.1 mCi/ml medium. Samples were then incubated for 1 h at the required light conditions. Subsequently, plants were washed in the dark with ice-cold distilled water, drained, and frozen. Degradation of the 32 kD protein was determined as follows: Plants were radiolabeled for 3 h in 30 μmol m^{-2} s^{-1} of cool-white fluorescent light and then distributed to 35 mm Petri dishes each containing 3 ml of chase medium (half-strength Hutner's medium with 1 mM methionine). After an additional hour in chase medium, in white light, the first time point (referred to as zero time chase) was taken. Plants were then incubated for 4, 9, and 18 h at the required light conditions. To stop the reactions, the medium was drained and the plants frozen. Membrane samples were prepared for SDS-PAGE as described by Marder et al. (16), and then assayed by fluorography. Loading of all gel lanes within a given experiment was done on an equal protein basis.

Light Sources. Cool-white fluorescent tubes were used for white light treatments. FR light was produced by filtering the light from a 500 W tungsten halogen floodlamp through a 180 mm thick water filter, a 2 mm thick KG3 (Schott) heat absorbing glass, and a layer of Wratten 89B filter (Kodak). The spectral properties of the output are shown in Figure 4A. Occasionally, an RG 715 filter glass (Schott), which gave a similar spectral photon distribution, was used in place of the Wratten 89B filter. L-FR light was produced by filtering the light from a projector equipped with a tungsten halogen lamp through heat absorbing glass and a sheet of Wratten 88A filter (Kodak) (Fig. 4A). Light measurements were made with a LiCor Li-188B quantum flux meter/radiometer and spectral photon distributions were measured with a LiCor 1800 spectroradiometer.

Delayed Luminescence. *Spirodela* fronds received a 100 ms flash of either white light (35 μmol m^{-2} s^{-1}) or FR light (70 μmol m^{-2} s^{-1} in the range 700–730 nm). PSII luminescence was detected by a photomultiplier through a 685 nm interference filter and a shutter timed to open 50 ms after the end of the light flash. The method is described by Malkin (15).

Photoacoustics. This technique directly measures the relative

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[5] Abbreviations: FR, far red; L-FR, long-wavelength far red; LHCP, light-harvesting Chl *a/b* protein.

evolution of O_2 from PSII in intact *Spirodela* fronds. Measurements were made according to Bults *et al.* (6).

Methyl Viologen Treatments. *Spirodela* fronds were incubated in 0.1 mM methyl viologen. Various light conditions, described in Table 1, were used to induce bleaching.

RESULTS

Metabolism of the 32 kD Protein in FR Light. Light ≥ 700 nm (FR light) activates PSI but not PSII, while light between 400 and 700 nm (white light) activates both photosystems (25). We compared the effects of FR light on synthesis and degradation of the 32 kD protein with those previously determined by Mattoo *et al.* (17) for white light and darkness. The results in Figure 1 show that darkness does not support a significant rate of 32 kD protein synthesis, while white light and FR light are both effective in this regard. By comparison, the LHCP is synthesized equally well under all three conditions. This is in agreement with previous data where synthesis of the LHCP was found to be initially uncoupled from illumination (4).
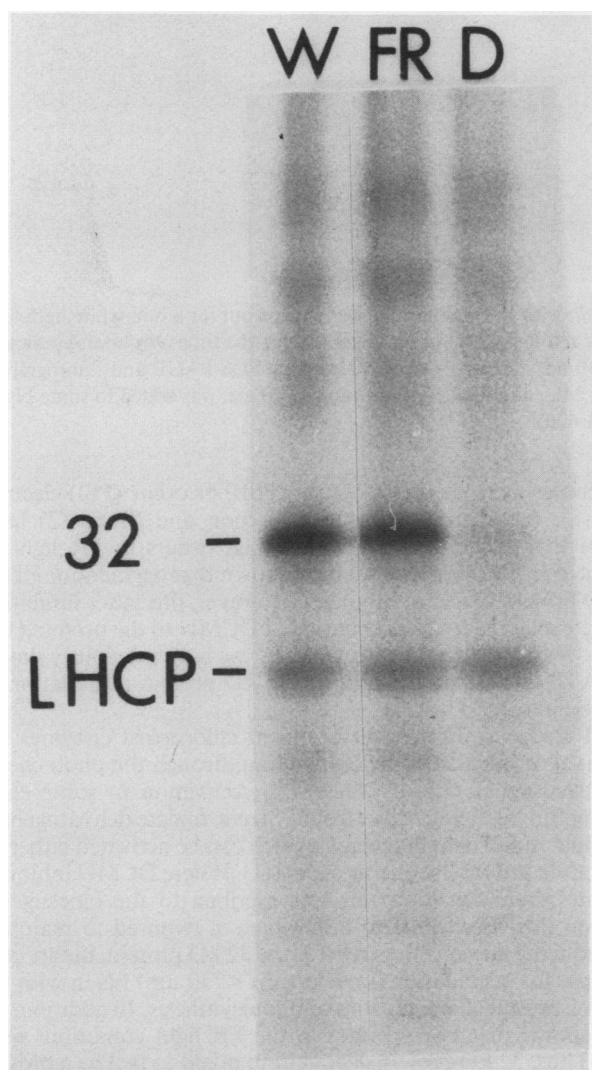

FIG. 1. Synthesis of the 32 kD protein in FR light. Radiolabeling with [35S]methionine was carried out for 1 h. Proteins were analyzed by SDS-PAGE and fluorography. Fluence rates in white light were 12 μmol m^{-2} s^{-1} and in FR light 50 μmol m^{-2} s^{-1} (in the range 700–730 nm). Spectral properties of the light sources are described in "Materials and Methods." W, white light; FR, far red light; D, darkness. The positions of the 32 kD protein and LHCP are indicated.

As seen in Figure 2, the 32 kD protein is also rapidly degraded in both white and FR light. Mattoo *et al.* (17) previously showed that in darkness, or in white light in the presence of DCMU, degradation of the 32 kD protein is considerably slowed. The results in Figure 2 reveal that DCMU likewise suppresses FR-light stimulated degradation of the 32 kD protein. The same was found with atrazine, another PSII inhibitor (data not shown). We note that in FR light (700–730 nm range) a fluence rate of 50 μmol m^{-2} s^{-1} was required to achieve a similar rate of 32 kD protein turnover as that obtained with 12 μmol m^{-2} s^{-1} of white light. Thus, while effective, FR light is less efficient than white light in driving the synthesis and degradation of this protein.

PSII Activity is Minimal under the FR Light Conditions Used. Metabolism of the 32 kD protein in FR light and the blockage of degradation by PSII-specific inhibitors were not anticipated. Therefore, it was important to establish whether both, or only one, of the photosystems were active *in vivo* under the FR light conditions used. Two separate tests were employed to determine PSII activity in FR light in *Spirodela* fronds. The first was the detection of delayed luminescence after a brief light pulse. Such light emission is known to be almost exclusively from PSII with very little contribution from PSI (15). As seen in Figure 3A, white light elicits a large response compared to the empty chamber control, whereas the FR light treatment produces a very low response.

Similar results were obtained when O_2 evolution was measured using the photoacoustic technique (Fig. 3B). Modulated 650 nm light produces a large, pulsed O_2 signal which is lost in the presence of the saturating (unmodulated) light. In contrast, there is very little O_2 evolution in response to the modulated FR light. We calculate that in intact *Spirodela* <3% of the O_2 is evolved in FR light (700–730 nm range) as compared with white light (400–700 nm) on an equal photon basis. When expressed on the basis of equal rates of 32 kD protein destruction under the two light regimes, this value is still <10%. Thus, PSII activity is minimal under the FR light conditions used.

Measurement of PSI-related Activity in FR Light. PSI activity is difficult to demonstrate directly in *Spirodela* fronds. We employed bleaching by light in the presence of methyl viologen as an indication of PSI activity in these plants. Methyl viologen is reduced by the terminal electron donor of PSI. Bleaching by light is considered to be caused by oxygen radicals which are formed in response to reoxidation of methyl viologen (7, 27). The data in Table I show that FR light (≥ 700 nm) induces bleaching of *Spirodela* in the presence of methyl viologen, although not quite as effectively as white light does. However, in L-FR light (>730 nm), where PSI activity is sharply reduced (25), Chl loss is minimal and comparable to the dark control. The source of electrons in *Spirodela* fronds for sustained reduction of methyl viologen in FR light is unknown.

The Absence of 32 kD Protein Degradation in Long-wavelength FR Light. Degradation of the 32 kD protein was examined using different broadband far red filters. The spectral characteristics of the light fields which were used are shown in Figure 4A. FR light promoted degradation of the 32 kD protein, while L-FR light was ineffective (Fig. 4B). Therefore, the 32 kD protein is stable under light conditions where both PSI and PSII are inactive.

DISCUSSION

We have demonstrated that degradation of the 32 kD protein in *Spirodela* fronds can occur as a function of FR light. We now discuss the means by which such light causes this process. Several alternatives are considered.

Phytochrome. There is the possibility that a nonphotosynthetic photoreceptor such as phytochrome might cause the FR light-induced responses shown here. This possibility is difficult to
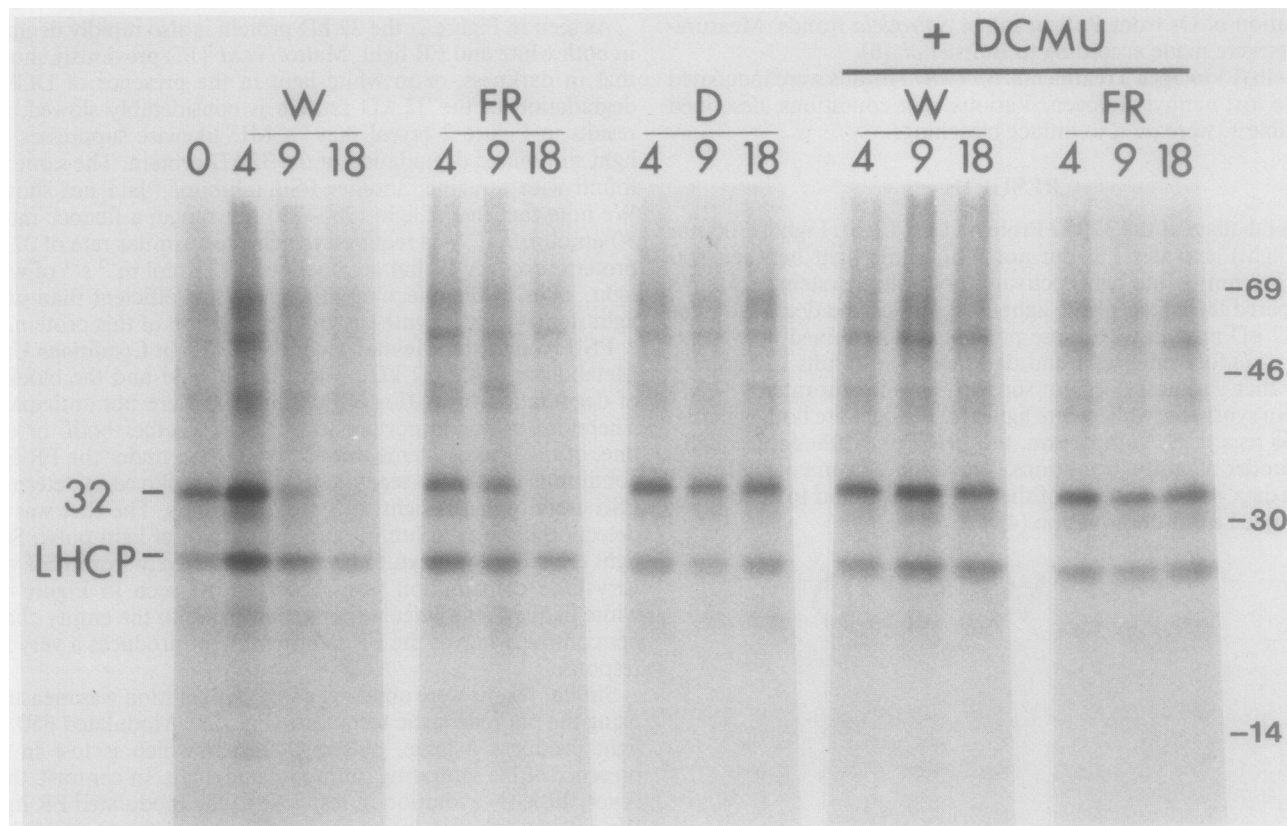
FIG. 2. Degradation of the 32 kD protein in FR light. Pulse labeling of *Spirodela* with [³⁵S]methionine was carried out for 3 h in white light. The radioactivity was then chased from fronds incubated in either white light (W), far red light (FR) or darkness (D) for the times (in hours) indicated above the gel lanes. The zero (0) time control is described in "Materials and Methods." Proteins were analysed by SDS-PAGE and fluorography. The fluence rates during the chase were as described in the legend to Figure 1. DCMU, at a final concentration of 10 μM, was added to some plants during the chase period as indicated. Mol wt markers were from Amersham (CFA 626).

exclude directly; however, it seems highly unlikely from our data. Continuous high-fluence rate FR light can elicit phytochrome responses in etiolated tissue, but these have not been found in green tissue such as we have used here (9, 26). There is also little possibility of a threshold response to phytochrome (21) in our case. The active form of phytochrome which is found in green plants is stable for many hours in darkness (13). Were it involved, it should be available to cause rapid degradation of the 32 kD protein in darkness. However, such degradation is known to occur at a minimal rate (17).

Plastoquinone Pool. A second alternative is that the reduction state of the plastoquinone pool, which modulates certain enzyme activities, could regulate 32 kD protein degradation. For example, a kinase which phosphorylates LHCP (29, 32) is controlled by the redox state of the plastoquinone pool. In broken chloroplasts, under aerobic conditions, added FR light reversed the phosphorylation process as it oxidized the plastoquinone pool (29). Similarly, FR light effectively reoxidized the plastoquinone pool in *Spirodela* fronds (JB Marder, unpublished data). However, in our case, degradation of the 32 kD protein can be activated under conditions where the plastoquinone pool is either reduced (*e.g.* in white light) or oxidized (*e.g.* in FR light). Moreover, in the dark, or in L-FR light, where degradation is deactivated, the plastoquinone pool is likewise oxidized. Therefore, it is unlikely that the redox state of the plastoquinone pool is involved in regulating the degradation of the 32 kD protein in *Spirodela.*

ATP Requirement. ATP is required for synthesis and, in some cases, degradation of proteins. In the chloroplast, the requirement

for synthesis can be met by linear (PSII) or cyclic (PSI) electron flow (8). As regards degradation, Arnon and Chain (2) have shown that cyclic electron flow, which occurs in FR light, is inhibited by DCMU; and we have shown that degradation of the 32 kD protein is also so inhibited. However, this latter inhibition can be explained by direct binding of DCMU to the protein (18). Moreover, by the use of an uncoupler, we have previously shown that ATP is not rate limiting for 32 kD protein degradation in white light (17).

PSI Reducing Power. A variety of chloroplast enzymes are activated or inactivated by light acting through the photochemical reactions of photosynthesis (5). Activation in some cases may require reducing power from PSI; *e.g.* malate dehydrogenase and glucose 6-P dehydrogenase, which can be activated either by light or via sulfydryl reducing agents (1). Where DCMU inhibited enzyme photoactivation this was ascribed to the blockage of electron flow from PSII to PSI, which is required to maintain PSI reducing power (5). As regards the 32 kD protein, the spectral response for degradation (wavelengths <730 nm) fits in with the known spectral characteristics of photosynthesis. In addition, we have shown that PSI operates in the FR light conditions used here. Thus, 32 kD protein degradation might in fact be a process activated by PSI reducing power. Degradation could involve activation of a specific protease or modification of the 32 kD protein to a degradation sensitive form. The inhibitory effect of DCMU on FR-driven 32 kD protein degradation would then be best understood as protection against protease degradation through direct binding of the inhibitor to the 32 kD protein molecule.
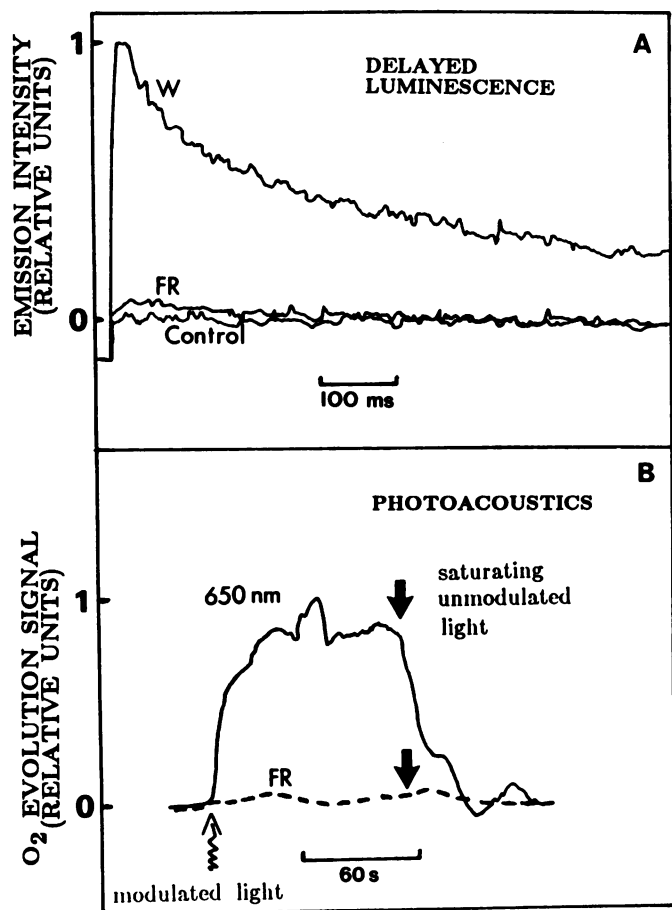
FIG. 3. Absence of PSII activity in FR light. A, Delayed luminescence. The relative intensity of the light emitted after a white light (W) or far red light (FR) pulse was measured as described in "Materials and Methods." The control is the luminescence of the empty sample compartment in FR light. B, Photoacoustic technique. Pulses of O_2 evolved from *Spirodela* fronds were measured in response to a modulated (chopped) light, either 650 nm or FR (≥ 700 nm). Fluence rates were 35 μmol m^{-2} s^{-1} at 650 nm and 70 μmol m^{-2} s^{-1} (in the range 700–730 nm) (FR). Measurements were made according to Bults *et al.* (6).

Table I. *Bleaching of Spirodela Fronds by Light in the Presence of Methyl Viologen*

Chl loss from intact plants was measured after 3 d of incubation in 0.1 mM methyl viologen. Chl content was determined by the method of Moran and Porath (22). Results are expressed as mean percentages of the control $\pm$ SE. Fluence rates were: white light (12 μmol m^{-2} s^{-1}), FR light (50 μmol m^{-2} s^{-1} in the range 700–730 nm), and L-FR light (80 μmol m^{-2} s^{-1}).

	% of Control[a]	n^b
White light	12 $\pm$ 1	9
FR light	24 $\pm$ 3	7
L-FR light	98 $\pm$ 8	3
Darkness	108 $\pm$ 10	10

[a] Control fronds incubated without methyl viologen were analyzed for Chl content under each light condition. [b] Number of repeats.

Semiquinone Anion. Another possible explanation for FR-light induced destruction of the 32 kD protein is related to its proposed role as a quinone binding protein. This explanation would extend the idea of Kyle *et al.* (14), who proposed that the Q_B semiquinone anion, formed transiently during PSII photoreactions, may inflict some damage on the 32 kD protein environment. The
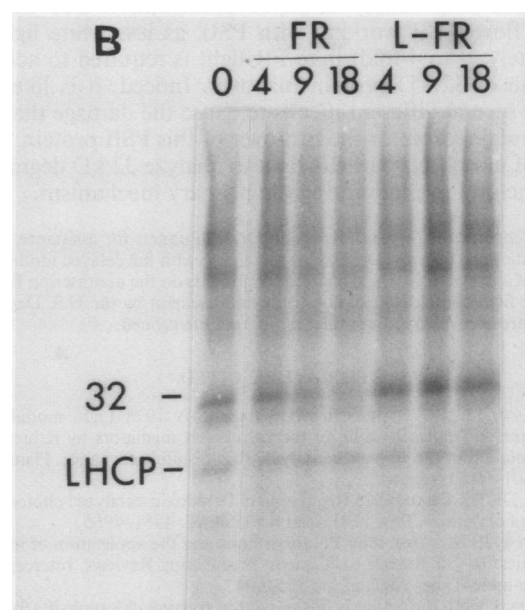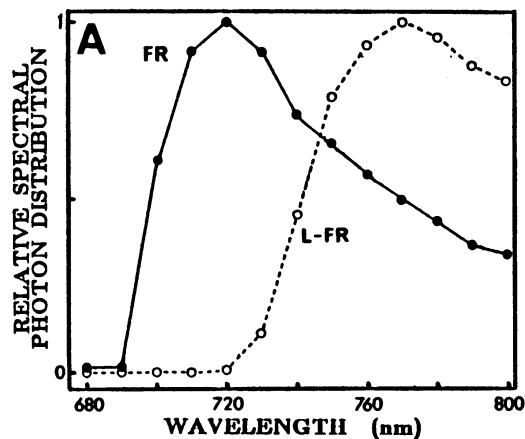

FIG. 4. A, Relative spectral photon distribution of the far red light sources used. FR light was generated using Wratten filter 89B as described in "Materials and Methods." Of the photons from the 700 to 800 nm waveband of the 89B source, 50% are in the range 700 to 730 nm. L-FR light was generated using Wratten filter 88A as described in "Materials and Methods." B, Spectral dependence of 32 kD protein degradation in far red light. The effects of the far red light sources, described in A, on 32 kD protein degradation were examined. The fluence rates in both cases were 80 μmol m^{-2} s^{-1} in the range 700 to 800 nm. Other experimental conditions are as described in the legend to Figure 2. A fluorograph following SDS-PAGE is shown.

proposal of Kyle *et al.* appears sufficient to explain degradation of the 32 kD protein in white light. DCMU inhibition of white-light driven degradation would be understood since the herbicide is thought to act by displacing Q_B from its binding environment (31). We suggest that FR light might also be expected to induce the formation of the Q_B semiquinone anion. Although photoreduction of plastoquinone is usually thought of as an activity of PSII, cyclic electron flow around PSI also seems to involve redox turnover of the plastoquinone pool (8). Since the plastoquinone reduction step in cyclic electron flow would necessarily involve transient semiquinone formation, there would be the continuous presence of potentially damaging semiquinone molecules even in FR light. Under these conditions DCMU would still protect

the 32 kD protein by preventing semiquinone anion binding. Thus, semiquinone-anion inflicted damage might be the activator for 32 kD protein degradation.

Free Radicals. Finally, it has been suggested that oxygen free radicals are generated during electron transport through PSI (11). It is possible that reactive compounds of this nature could cause localized damage to the 32 kD protein resulting in its degradation. Free radicals would also be generated when PSII and PSI are operating together (12, 28), and this as well could be the unifying theme for degradation in FR and white light.

In summary, the mechanism of light-dependent 32 kD protein turnover is still unclear. An *in vitro* 32 kD protein degradation system (24), amenable to both light and chemical activation, could aid in distinguishing among the various possibilities. However, for a cell-free system to be useful, equivalence with the *in vivo* situation will have to be proved. Although we can conclude that FR light can cause turnover of the 32 kD protein, it would be erroneous to assume that this is the only control operating *in vivo*. There is probably a major contribution due to linear electron flow (PSII working with PSI), as less white light (approximately 3- to 4-fold) than FR light is required to achieve a given rate of 32 kD protein turnover. Indeed, it is likely that there are several different means to cause the damage that is the fundamental reason for the turnover of this PSII protein. Applications of nonphotosynthetic light to analyze 32 kD degradation may be helpful in pinpointing the primary mechanism.

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