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Ultraviolet B Exposure of Whole Leaves of Barley Affects Structure and Functional Organization of Photosystem II*

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This study examines the effects of ecologically important levels of ultraviolet B radiation on protein D1 turnover and stability and lateral redistribution of photosystem II. It is shown that ultraviolet B light supported only limited synthesis of protein D1, one of the most important components of photosystem II, whereas it promoted significant degradation of proteins D1 and D2. Furthermore, dephosphorylation of photosystem II subunits was specifically elicited upon exposure to ultraviolet B light. Structural modifications of photosystem II and changes in its lateral distribution between granum membranes and stroma-exposed lamellae were found to be different from those observed after photoinhibition by strong visible light. In particular, more complete dismantling of photosystem II cores was observed. Altogether, the data reported here suggest that ultraviolet B radiation alone fails to activate the photosystem II repair cycle, as hypothesized for visible light. This failure may contribute to the toxic effect of ultraviolet B radiation, which is increasing as a consequence of depletion of stratospheric ozone.

An increased level of ultraviolet B radiation (280–320 nm) reaching the Earth's surface has been observed as a consequence of depletion of stratospheric ozone. This phenomenon, first described over the Antarctic Circle, is now extending significantly even to temperate regions (1). It has been shown that even low levels of ultraviolet B light may harm most biological organisms, land plants being particularly sensitive to this radiation (2, 3). The molecular basis of ultraviolet B-induced damage is not completely understood. Apart from DNA damage (4) and other effects such as activation of genes involved in the phenylpropanoid pathway (5, 6), ultraviolet B light impairs photosynthesis. PSII[1] is the most sensitive protein complex of the photosynthetic electron transfer chain (7–9). Damage to the manganese cluster associated with oxygen-evolving activity (10–12), redox-active tyrosines (12, 13), P_{680} (the primary donor in photosystem II) (14), and bound (15, 16) and unbound (10, 15) plastoquinone molecules has been reported. As a consequence of damage, loss of photosynthesis and degradation of reaction center proteins D1 and D2 occur. Studies carried out using *in vitro* systems have shown that irradiation with ultraviolet B light brings about a loss of protein D1, paralleled by the appearance of an immunodetectable C-terminal fragment of 20 kDa (17). This fragment is produced by cleavage in the second transmembrane α-helix (17) in a reaction depending on the presence of manganese ions bound at the catalytic site(s) of the PSII donor side (10). Degradation of protein D2 upon ultraviolet B exposure occurs both *in vitro* (18) and *in vivo* (19), although the appearance of specific degradation fragments of protein D2 has so far been reported only *in vitro* (18). Since protein cleavage was observed only when the Q_A site was at least partially active, a role for the bound quinone in the ultraviolet B-induced degradation of protein D2 was inferred. Recently, the possible role of protein phosphorylation in ultraviolet B-mediated degradation of proteins D1 and D2 has been proposed, suggesting the possible presence of proteolytic activity involved in the degradative process (20).

A feature that makes PSII unique among membrane protein complexes is the rapid light-dependent turnover of its reaction center subunit, D1 (21). Degradation of protein D1 induced by visible light is known to affect both the structural organization and lateral distribution of PSII centers (22–24). These modifications are thought to be part of a repair cycle in which damaged centers migrate from granum stacks to stroma-exposed membranes, where insertion of newly synthesized protein D1 takes place (for a review, see Ref. 25). Synthesis of protein D1 is regulated mainly at the translational level, through the thioredoxin system and ADP-dependent protein phosphorylation (26). However, at least in cyanobacteria, the synthesis and degradation of protein D1 are synchronized, indicating interplay between the two phenomena (27). In addition, it has recently been shown that in higher plants such as *Spirodela oligorhiza*, pumpkin, and spinach, reversible phosphorylation of protein D1 plays an important role in the regulation of this degradative process (28–30), dephosphorylation of protein D1 being a compulsory step in its degradation pathway (28). Reversible phosphorylation of other PSII polypeptides (CP43, D2, PsbH) is believed to be involved in coordinating dismantling of damaged centers and their lateral redistribution (31). Whether degradation of reaction center proteins induced by ultraviolet B light alone or in combination with visible light has the same effect on PSII structure and lateral distribution is currently unknown. In cyanobacteria, the loss of the dimeric form of PSII induced by ultraviolet B has been shown (32). However, selective dissociation of the inner antenna CP43, mediated by degradation of protein D1, was not detected. Instead, full dismantling of PSII was observed. Whether this is a general effect of ultraviolet B light or a peculiarity of these organisms, in which

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[1] The abbreviations used are: PSII, photosystem II; Tricine, *N*-tris(hydroxymethyl)methylglycine; PAGE, polyacrylamide gel electrophoresis; LHCII, light harvesting complex of photosystem II.

phosphorylation of core proteins and lateral differentiation of thylakoids are lacking (33, 34), is still an open question.

In this study, we report that ultraviolet B light affects protein D1 turnover as well as PSII stability and lateral redistribution, with significant differences with respect to what has been observed under photoinhibitory conditions by visible light. Throughout this study, we used 10 μmol·m^{-2}·s^{-1} ultraviolet B light, an intensity chosen because its ecological importance is similar to that detected under natural conditions. The intensity of the control visible light (100 μmol·m^{-2}·s^{-1}) was sufficient to drive photosynthesis without photoinhibitory effects.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—Kernels of barley (*Hordeum vulgare* L.) were soaked in tap water for some hours, planted in a mixture of autoclaved vermiculite/garden soil, and vernalized for 48 h at 4 °C. Plants were grown for 7–10 days at 22 °C, with a light intensity of 100 μmol·m^{-2}·s^{-1} and a photoperiod of 12 h. Relative humidity was 70%.

Irradiation of Plants—Barley leaves were cut and floated in water. Detached leaves were irradiated using a Vilbert-Lourmat 215M lamp as an ultraviolet B source. The lamp was wrapped in cellulose diacetate foil (0.15-mm thick) to screen out any ultraviolet C component emitted by the ultraviolet B source. Light intensity at the sample surface was 10 μmol·m^{-2}·s^{-1}, as measured with a Skye UV-B sensor.

Thylakoid Isolation and Digitonin Subfractionation—For thylakoid isolation, plants were frozen in liquid nitrogen, homogenized with a mortar and pestle to a fine powder, and ground in grinding buffer (50 mM Tricine (pH 8.0), 5 mM $MgCl_2$, 10 mM NaF, and 0.33 M sorbitol). After filtering through eight layers of cotton gauze, thylakoids were pelleted by centrifugation for 5 min at 4500 × *g*. Pellets were resuspended in grinding buffer without sorbitol. Thylakoids were again pelleted and then resuspended in grinding buffer. The activity of thylakoids, as measured by oxygen evolution in the presence of 0.5 mM 2,5-dimethyl-*p*-benzoquinone as an electron acceptor, was in the range of 100–140 μmol of O_2·mg of chlorophyll^{-1}·h^{-1}. For digitonin fractionation (35), thylakoids were made up to 0.4 mg of chlorophyll·ml^{-1}, and digitonin was added to a final concentration of 0.5% (w/v). This suspension was incubated for 30 min in ice with stirring and then diluted with 10 volumes of digitonin-free buffer. Unsolubilized material was eliminated by centrifugation for 5 min at 4000 × *g*, and granum-, margin-, and stroma-enriched fractions were isolated by centrifugation at 10,000 (10 min), 40,000 (30 min), and 100,000 (90 min) × *g*, respectively.

Sucrose Gradient Centrifugation—Thylakoids were resuspended in sucrose-free buffer, and *n*-dodecyl β-D-maltoside was added to a final concentration of 1% (w/v). After 5 min of incubation on ice, unsolubilized material was removed by centrifugation for 5 min at 13,000 ×*g*, and the supernatant (equivalent to 60 μg of chlorophyll) was loaded on the top of a 4-ml 0.1–1.0 M sucrose gradient made up in 10 mM Tricine (pH 7.8), 10 mM NaF, and 0.03% *n*-dodecyl β-D-maltoside. Gradients were spun for 5 h at 56,000 rpm in a Kontron TST 60.4 rotor at 4 °C. After centrifugation, sucrose gradients were fractionated into 20 aliquots (200 μl each), each of which was subjected to SDS-PAGE and immunoblotting (see below).

Urea/SDS-PAGE and Immunoblotting—SDS gel electrophoresis in the presence of 6 M urea was carried out as described previously (36). For immunoblotting, proteins resolved by SDS-PAGE were again transferred electrophoretically onto polyvinylidene difluoride membranes using the carbonate/bicarbonate-based buffer described in Ref. 37. Blots were immunodecorated as described elsewhere (21, 35). The properties of polyclonal antibodies to different PSII subunits and LHCII used in this study have been described previously (22). Polyclonal antibodies to phosphothreonine-containing proteins were from Zymed Laboratories Inc. (South San Francisco, CA) and were used as described (38). Immunoreactions were detected using a chemiluminescence kit (either New England Biolabs Inc. or Pierce SuperSignal for alkaline- or peroxidase-conjugated secondary antibodies, respectively) and were visualized by autoradiography using Fuji RX as detection film. Densitometric analysis of autoradiographic films was performed with a GelDoc 1000 system and Molecular Analyst software (Bio-Rad).

In Vivo Labeling with [^{35}S]Methionine—For *in vivo* labeling, barley leaves were cut into 0.5-cm segments and incubated in a solution containing 100 μCi/ml [^{35}S]methionine. For synthesis experiments, labeling was carried out for either 3 or 6 h under the following irradiation conditions: dark, ultraviolet B light (10 μmol·m^{-2}·s^{-1}), and white light (100 μmol·m^{-2}·s^{-1}). For degradation experiments, labeling was instead carried out under visible light for 90 min as described above. Leaves were then incubated in 1 mM unlabeled methionine for the desired periods of time under different irradiation conditions: dark, ultraviolet B light (10 μmol·m^{-2}·s^{-1}), and visible light (100 μmol·m^{-2}·s^{-1}) for 2, 4, 8, and 22 h. The temperature was 24 °C. After urea/SDS-PAGE, gels were stained with 0.1% (w/v) Coomassie Blue R-250 in methanol/water/acetic acid (5:5:1 by volume) and destained in 7.5% acetic acid and 10% methanol. Before vacuum-drying, gels were soaked in Amplify (Amersham Pharmacia Biotech) for 1 h and then subjected to fluorography. 7 μg of chlorophyll were applied to each gel lane. A second gel (containing 1 μg of chlorophyll/lane) was blotted onto polyvinylidene difluoride. The filter was subjected to autoradiography and then probed with antibody to protein D1 or other PSII subunits.

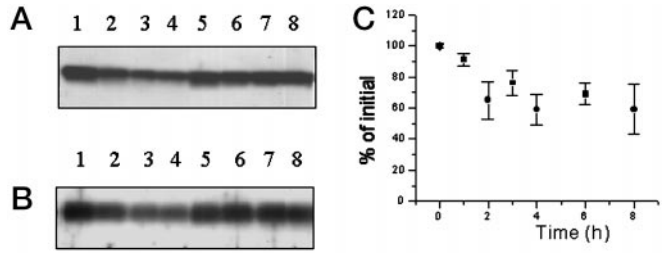

FIG. 1. *A* and *B*, levels of reaction center proteins D1 and D2, respectively. Levels of reaction center proteins were determined by immunoblotting with monospecific polyclonal antibodies using thylakoids isolated from leaves irradiated with ultraviolet B (*lanes 1–4*) or white light (*lanes 5–8*) for 0 h (*lanes 1* and *5*), 1 h (*lanes 2* and *6*), 3 h (*lanes 3* and *7*), and 6 h (*lanes 4* and *8*). Each gel lane contained 0.1 μg of chlorophyll. *C*, densitometric analysis of protein D1 shown in *A* (*lanes 1–4*) (*circles*) and F_V/F_M ratio measured on whole leaves (*squares*). *Error bars* represent S.D. for each point, with $n = 4$ for densitometry and $n = 20$ for F_V/F_M values. Both parameters remained unchanged after 8 h in control (non-irradiated) leaves. See "Experimental Procedures" for other conditions.

Electron Microscopy—Samples from differently irradiated leaves were fixed overnight at 4 °C in 3% glutaraldehyde and 0.1 M sodium cacodylate buffer (pH 6.9) and then processed for electron microscopy according as described (39). Ultrathin sections, cut with an ultramicrotome (Ultracut, Reichert-Jung), were observed with a transmission electron microscope (TEM 300, Itachi) operating at 75 kV.

Chlorophyll Fluorescence—Chlorophyll fluorescence analyses were performed with a PAM 101 fluorometer (Waltz).

RESULTS

Levels of Polypeptides D1 and D2 Are Specifically Affected by Ultraviolet B Light—The steady-state levels of reaction center proteins D1 (*panel A*) and D2 (*panel B*) after different periods of exposure of the leaves to ultraviolet B light (*lanes 1–4*) are shown in Fig. 1. A quantitative estimate by densitometric analysis of the immunoblot in *panel A* is given in *panel C* (*circles*). As previously reported, polypeptides D1 and D2 are particularly sensitive to ultraviolet B radiation (17, 19). Under our experimental conditions, the steady-state levels of both reaction center proteins decreased to ~45% of the original value after 8 h of irradiation (*panels A* and *B*, *lanes 1–4*; see also Ref. 20). The steady-state levels of other PSII core polypeptides such as CP47 and CP43 were not significantly affected under these conditions (data not shown). No variation in the level of any thylakoid polypeptide was observed upon irradiation with visible light at the intensity used in this study (100 μmol·m^{-2}·s^{-1}), as shown in the case of proteins D1 and D2 (*lanes 5–8*).

The loss of PSII activity (as measured by the F_V/F_M ratio) is also reported in Fig. 1*C* (*squares*). The decrease in the protein D1 level was correlated in time and amplitude with the loss of PSII activity. The apparent faster decay of protein D1 is not significant, as the difference is within the experimental error. The result is relevant because during photoinhibition by visible light, the level of protein D1 decays at a much lower rate than photosynthetic activity (9).

Protein D1 Turnover—In principle, the loss of the steady-

state level of reaction center proteins observed upon exposure to ultraviolet B light may be due to enhanced degradation, reduced synthesis of the protein, or to both phenomena together. To investigate this point, two types of labeling experiments were performed: (i) labeling of whole leaves during irradiation with either visible or ultraviolet B light and (ii) [^{35}S]methionine labeling of leaves under visible illumination and chase of radioactivity under either visible or ultraviolet B light. Control plants were kept in the dark under the same conditions in the presence of the label.

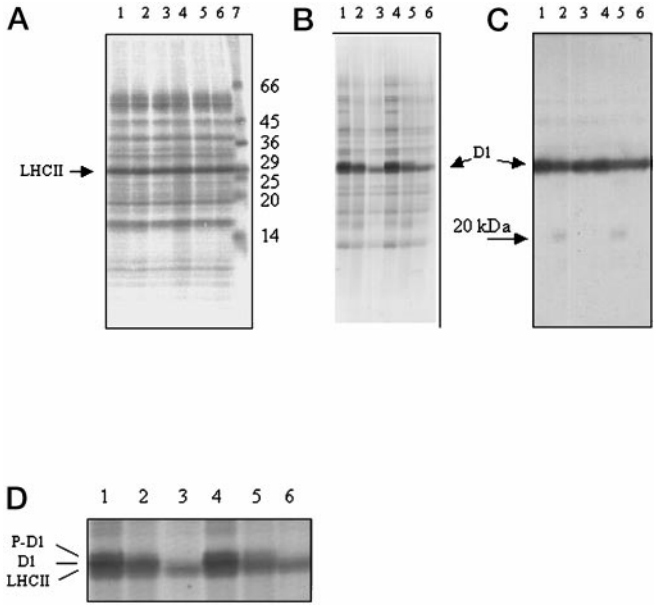

FIG. 2. **Incorporation of radioactivity into thylakoid polypeptides during irradiation with ultraviolet B or white light.** Thylakoids isolated from barley leaves incubated with 100 μCi/ml [^{35}S]methionine under visible light (*lanes 1* and *4*) or ultraviolet B light (*lanes 2* and *5*) or in the dark (*lanes 3* and *6*) were loaded on SDS-polyacrylamide gels. *A*, Coomassie Blue-stained gel (7 μg of chlorophyll/lane). *B*, autoradiography of a blotted gel identical to that shown in *A* but with 1 μg of chlorophyll/lane. *C*, immunoblot with antibody to protein D1 of the filter shown in *B*. Samples in *lanes 1–3* and *4–6* contained thylakoids isolated from leaves incubated with radioactive methionine for 3 and 6 h, respectively. *D*, magnification of the 35–25-kDa region of the autoradiograph shown in *B*. *Lane 7* of *A* contained molecular mass markers. *P-D1* in *D* refers to the protein D1 precursor.

Results from the first type of experiment in which barley leaves were labeled are shown in Fig. 2. *Panel A* shows a Coomassie Blue-stained gel: no significant differences were observed between the samples under the various conditions. A gel containing the same samples as in *panel A*, but with only 1 μg of chlorophyll (instead of 7) to avoid saturation, was blotted onto polyvinylidene difluoride membrane and, before immunodetection, subjected to autoradiography (*panel B*). It was apparent that on a chlorophyll basis, the samples labeled under ultraviolet B light (*lanes 2* and *5*) incorporated less radioactivity than the samples labeled under white light (*lanes 1* and *4*). Samples incubated with the label in the dark contained some radioactivity at the level of LHCII (*lanes 3* and *6*), but not at the level of protein D1. Densitometric evaluation of several autoradiographic films similar to that in *panel D* indicated that incorporation of radioactivity under white light was higher than that under ultraviolet B by a factor of ~2. In the magnification shown in *panel D*, the different positions of LHCII (lower portion of the band) and protein D1 (upper portion) are clearly evident. It may be observed that (i) only the LHCII fraction is significantly labeled in the dark (*lanes 3* and *6*); (ii) LHCII labeling is essentially the same in the various lanes; and (iii) under both ultraviolet B and visible light, incorporation of radioactivity in protein D1 is already saturated after 3 h of incubation with the label.

An immunoblot probed with antibody to protein D1 is shown in Fig. 2*C*. According to the data reported above, some 30% of total protein D1 was lost during irradiation, and the typical 20-kDa C-terminal D1 breakdown fragment was detected (*lanes 2* and *5*).

Results from the second type of experiment are shown in Fig. 3. It was possible to compare directly the degradation rate of

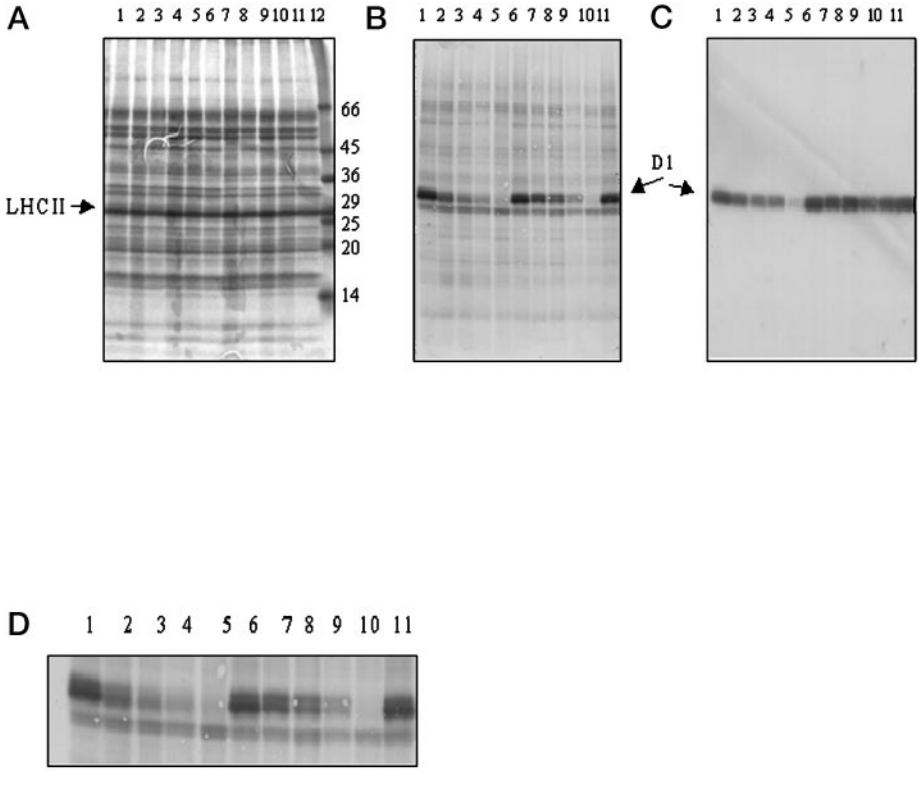

FIG. 3. **Loss of radioactivity from ^{35}S-labeled thylakoid polypeptides during chase under different irradiation conditions.** After labeling with [^{35}S]methionine, leaves were chased in ultraviolet B light (*lanes 1–5*) or white light (*lanes 6–10*) or were kept in the dark (*lane 11*) for 0 h (*lanes 1* and *6*), 2 h (*lanes 2* and *7*), 4 h (*lanes 3* and *8*), 8 h (*lanes 4* and *9*), and 22 h (*lanes 5*, *10*, and *11*). *A*, Coomassie Blue staining. *B*, autoradiography. *C*, immunoblotting with anti-D1 polyclonal antibody. Other conditions were as described in the legend to Fig. 2. *D*, magnification of the 35–25-kDa region of the autoradiograph shown in *B*.

protein D1 under the two irradiation conditions in this experiment. *Panel A* shows a Coomassie Blue-stained gel: no significant differences were observed between samples. From the autoradiography results shown in *panel B*, it can be seen that ultraviolet B light promoted faster degradation of protein D1 than visible light, by a factor of ~2. This was evaluated from densitometric analysis of the magnification of the protein D1 lane, as reported in *panel D*. The immunoblot (*panel C*) shows the expected loss of the steady-state level of protein D1, which paralleled the loss of radioactivity (*lanes 1–5*). After 22 h of exposure to ultraviolet B, protein D1 was barely detectable, either by autoradiography (*panel B*, *lane 5*) or immunoblotting (*panel C*, *lane 5*). Instead, the level of protein D1 under visible light, both as radioactive protein (*panel B*, *lane 11*) and total protein (*panel C*, *lanes 6–11*), remained constant.

Structural Changes in Photosystem II—The next question we posed was whether and to what extent degradation of proteins D1 and D2 affects the structural organization of PSII centers. To answer this question, thylakoids isolated from plants irradiated for 3 and 6 h were solubilized with *n*-dodecyl β-D-maltoside and fractionated by sucrose gradient centrifugation. This technique allows the resolution of the thylakoid membrane into the main photosynthetic complexes, preserving the oligomeric structure of the different complexes if solubilization conditions are carefully controlled. As reported previously by us (22, 31) and others (41), four pigmented bands were resolved under our experimental conditions. These were named B1 to B4 in order of increasing mobility. On the basis of optical properties (absorption and fluorescence spectroscopy), polypeptide content, and reactivity with monospecific polyclonal antibodies, these bands have been identified as monomeric (B1) and trimeric (B2) LHCII, monomeric PSII core complex (B3), and monomeric PSI superimposed on dimeric PSII (B4). It has previously been shown that variation in the supramolecular organization of photosynthetic complexes following light stress can easily be detected by the combination of sucrose gradient centrifugation and immunoblotting (22, 31). For example, a transition from the dimeric to monomeric form of PSII may be seen as a shift of immunoreactivity from fractions corresponding to the bottom (where the dimeric form migrates) to fractions corresponding to the middle (where the monomeric form is located); dissociation of CP43 from the core of PSII is detected as an increase in immunoreactivity in fractions corresponding to a upper region of the sucrose gradient. According to this scheme, thylakoids isolated from control and ultraviolet B-treated plants were isolated, solubilized with *n*-dodecyl β-D-maltoside, and fractionated by sucrose gradient centrifugation as described above. Gradients were fractionated into 20 aliquots (200 μl each), loaded onto a 20-well gel, and subjected to SDS-PAGE and immunoblotting as shown in Fig. 4. *Lane 1* contains unfractionated thylakoids, whereas *lanes 2–19* contain the respective fractions from the sucrose gradients (from top to bottom). Therefore, *lanes 2–8* correspond to green bands B1 and B2 (*i.e.* antenna and other monomeric proteins); *lanes 9–12* correspond to band B3 (monomeric PSII core); and *lanes 14–18* correspond to band B4 (*i.e.* dimeric PSII and monomeric PSI). When probed with antibodies to PSII proteins (a mixture of anti-CP47, anti-D2, and anti-D1 antibodies) two immunoreactive peaks, corresponding to monomeric (fractions 9–12, band B3) and dimeric (fractions 14–18, band B4) PSII cores, were observed with thylakoids from control leaves (*panel A*). The same kind of distribution was obtained when antibodies to other PSII subunits such as anti-CP43 and anti-cytochrome b_{559} α-subunit antibodies were used (data not shown). Upon ultraviolet B irradiation, the intensity of immunoreactions corresponding to the dimeric form did not decrease (*panels B* and *C*). These results indicate that, as far as ultraviolet B irradiation is concerned, inactivation of PSII centers, as evaluated by loss of variable fluorescence and degradation of proteins D1 and D2, does not induce monomerization of the centers.

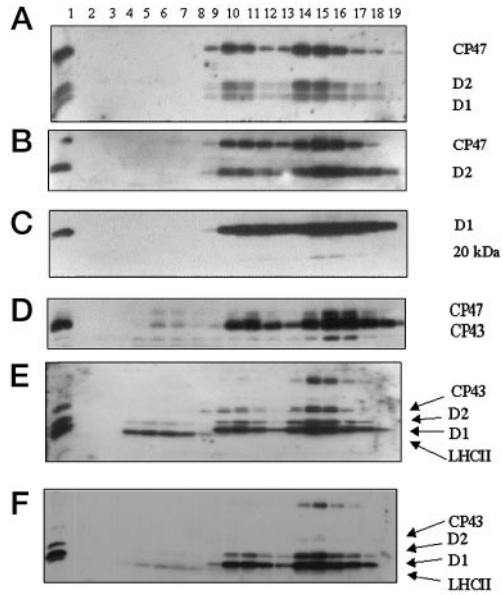

FIG. 4. **Fractionation by sucrose gradient centrifugation of thylakoids isolated from leaves treated with ultraviolet B for 0, 3, or 6 h.** Thylakoids from control (*A* and *E*) and ultraviolet B-treated (*B–D* and *F*) plants were fractionated by sucrose gradient centrifugation as described under "Experimental Procedures." After centrifugation, gradients were fractionated into 200-μl aliquots, each of which was analyzed by immunoblotting with the indicated antibodies. *Lane 1* in the different panels contained thylakoids before gradient fractionation (0.25 μg of chlorophyll), whereas *lanes 2–19* contained the respective sucrose gradient fractions (from top to bottom). 25 μl of each fraction were loaded per lane. Fractions 2–8 therefore correspond to sucrose gradient green bands B1 and B2, fractions 9–12 to green band B3 (*i.e.* monomeric PSII), and fractions 13–19 to green band B4 (dimeric PSII superimposed on monomeric PSI). *A*, control thylakoids probed with a mixture of antibodies to CP47 and proteins D2 and D1; *B*, thylakoids from leaves irradiated for 3 h with ultraviolet B and probed with antibodies to CP47 and protein D2; *C*, thylakoids from leaves irradiated for 6 h and probed with antibodies to protein D1; *D*, same as *C*, but probed with antibodies to CP47 and CP43; *E*, control thylakoids probed with polyclonal antibodies to phosphothreonine-containing proteins; *F*, thylakoids from leaves irradiated for 3 h with ultraviolet B probed with antibodies to phosphothreonine-containing proteins.

Using the same procedure based on sucrose gradient fractionation and immunoblotting, we found that the 20-kDa C-terminal D1 fragment was present in both bands B3 and B4 (Fig. 4*C*). In the same sucrose gradient bands, polypeptide D2, CP43, CP47, the cytochrome b_{559} subunit, and intact protein D1 were also found (data not shown). No breakdown fragments were observed in fractions from the top of the gradients, which contained free-running proteins. These data indicate that inactivation of centers and cleavage of protein D1 do not induce release of any damaged protein. It should be noted that according to previous data on the effect of high visible light (22, 31), some free CP43 was observed in the top fractions of our sucrose gradients (*panel D*, *lanes 6* and *7*). However, at variance with those reports, some free-running CP47 was also detected (*panel D*, *lanes 6* and *7*), indicating that full dismantling of a fraction of PSII centers occurs as a consequence of extensive damage to PSII induced by ultraviolet B light.

Polyclonal antibodies to phosphothreonine-containing proteins, which recognize the phosphorylated forms of LHCII, proteins D1 and D2, and CP43, indicated that the level of phosphorylation in dimeric and monomeric forms of PSII was equivalent, as it was roughly proportional to the intensity of

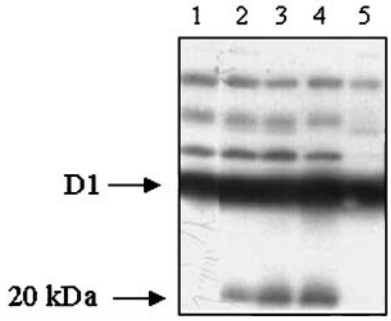

FIG. 5. **Location of D1 breakdown fragments in different membrane compartments.** *Lanes 1* and *2*, thylakoids from non-irradiated and irradiated leaves, respectively; *lanes 3–5*, granum, margin, and stroma-exposed lamella membrane fractions from irradiated leaves, respectively. Immunoblotting was carried out using anti-D1 antibody specific for the C terminus of protein D1.

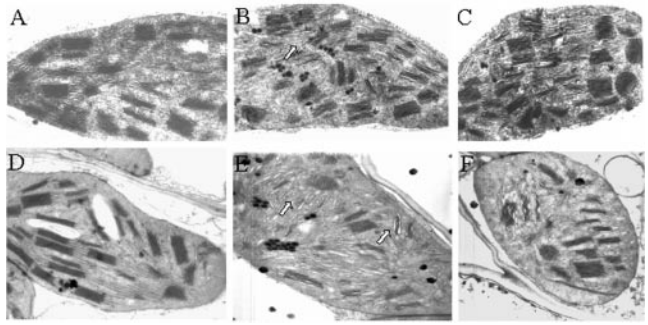

FIG. 6. **Electron micrographs of barley leaves treated with ultraviolet B light for 0 h (*A* and *D*), 3 h (*B* and *E*), and 6 h (*C* and *F*).** *A–C*, samples from the wild type; *D–F*, samples from the chlorophyll *b*-less mutant *chlorina f2*. *Arrows* indicate vesiculation of grana.

the observed immunoreaction in the same fractions with monospecific antibodies to different PSII proteins (compare Fig. 4, *panels A* and *E*). It should also be noted that free CP43 was never detected as a phosphorylated protein (*panel E*), suggesting that its dissociation from the core requires dephosphorylation. No additional low molecular mass polypeptides appeared as a consequence of ultraviolet B irradiation, indicating that breakdown of proteins in their phosphorylated form does not occur. Moreover, it may be noted that after ultraviolet B light exposure, almost complete dephosphorylation of LHCII and CP43 proteins was found in the blot from the sucrose gradient (compare *panels E* and *F*).

Lateral Redistribution of Photosystem II—To gain additional insight on the effect of ultraviolet B light on the lateral distribution of PSII, thylakoids from irradiated plants were fractionated into granum-, margin-, and stroma-enriched fractions by the digitonin method (35). As a consequence of irradiation with ultraviolet B light, a decrease in the chlorophyll a/b ratio was observed in stroma membranes, from 5.2 for the control to 4.4 for the irradiated sample. Immunoblotting with antibodies to protein D1 clearly shows that breakdown fragments of the protein, detected in thylakoids (Fig. 5, *lane 2*), were located in the granum and margin fractions (*lanes 3* and *4*). No trace of these fragments could be observed in the digitonin-derived stroma-exposed membrane fraction (*lane 5*). This finding is at variance with what is known about the lateral redistribution of PSII centers during photoinactivation with white light. In the latter case, upon degradation of protein D1, damaged centers migrate to stroma-exposed membranes (22, 31), where the damaged protein D1 is substituted with a newly synthesized copy (42). Fig. 6 (*panels A–C*) shows electron micrographs of barley leaves irradiated with ultraviolet B light for different periods of time. Treatment with ultraviolet B light apparently brought about general swelling of thylakoid membranes, which, in some cases (emphasized by *arrows* in the 3-h irradiated samples, but clearly visible also after 6 h), could be considered as partial destacking of the membrane. We interpret this phenomenon as the consequence of the dismantling of a fraction of those PSII cores that are involved in membrane stacking (43–45). This interpretation is supported by the observation that this phenomenon was even more pronounced when the chlorophyll *b*-less mutant *chlorina f2* was treated in the same way with ultraviolet B light (*panels D–F*). In this mutant, stacking of thylakoids is thought to be maintained mainly by PSII (43) and only marginally by residual LHCII (43, 44). Thus, in view of this result, we interpret the increase in LHCII observed in the digitonin-derived stroma-exposed membranes of the wild type as the result of more extensive solubilization of membranes, produced by the detergent because of partial destacking linked to PSII degradation rather than to actual lateral migration of antenna proteins. In fact, ultraviolet B-stimulated dephosphorylation of LHCII polypeptides is a condition expected to retain LHCII polypeptides in the granum membranes rather than to promote their migration into stroma-exposed lamellae.

DISCUSSION

As a consequence of ultraviolet B irradiation of whole leaves, a pronounced decrease in the steady-state levels of reaction center proteins D1 and D2 was observed. The levels of other PSII core subunits such as CP47, CP43, and the α-subunit of cytochrome b_{559}, as well as those of more peripheral polypeptides such as LHCII, did not significantly change. These findings have a number of implications at the level of metabolism of protein D1 and hence in the supramolecular organization and lateral redistribution of PSII complexes.

In the case of protein D1, loss of the protein level may be deduced semiquantitatively from densitometric analysis (Fig. 1). This shows that under ultraviolet B radiation, a new level is reached when ~50% of protein D1 is degraded in 4 h. A steady-state level under irradiation is determined by the interplay between the rate of degradation of the protein, a first-order process, and the rate of synthesis, a process that may be considered, at a first approximation, as zero order, independent of the concentration of protein D1. However, evaluation of the effect of ultraviolet B radiation on the actual steady-state level of protein D1 is made difficult by the fact that penetration of ultraviolet B radiation through the leaves is not complete and that only a fraction of the reaction centers are actually reached by the radiation. Under our experimental conditions, the degradation of protein D1, as measured by pulse-chase experiments with [^{35}S]methionine, shows time constants of 4 h under ultraviolet B radiation and of 8 h under visible light (see Fig. 3). The rate of synthesis, as judged by incorporation of [^{35}S]methionine under ultraviolet B radiation, is lower by a factor of ~2 with respect to that observed under illumination with visible light (see Fig. 2). Thus, ultraviolet B radiation at the intensity used here increases degradation and inhibits synthesis of protein D1 with respect to non-photoinhibitory visible light. After more prolonged ultraviolet B irradiation, more protein D1 is degraded, showing that under these conditions, a real steady state for this protein is not attained (see Fig. 3).

The increased degradation of protein D1 under ultraviolet B radiation may be explained as the consequence of two independent mechanisms. The first is direct damage to the donor side through impairment of the manganese cluster (11, 12) or

other donor-side components (12–14), which leads to cleavage of the protein in the second transmembrane helix (17). The second mechanism may be ascribed to induced dephosphorylation of PSII proteins, which favors turnover of protein D1, according to the model proposed by Aro and co-workers (38). The latter dephosphorylation action of ultraviolet B is evident in Fig. 4, where almost complete dephosphorylation of LHCII and CP43 is observed, and has also been previously described (20) for protein D1. The inhibitory effect of ultraviolet B light on the synthesis of protein D1 is more difficult to explain. According to current models (26), in green algae and higher plants, regulation of protein D1 synthesis occurs mainly at the translational level through the binding of a complex of four proteins (RB60, RB55, RB47, and RB38) to the 5′-untranslated region of D1 mRNA. Binding activity is greater in illuminated cells than in dark-adapted cells, suggesting that redox control may be important for regulation of translational activity. It is therefore possible that ultraviolet B light, which supports oxygen evolution only to a limited extent (16), cannot efficiently activate redox-controlled translation of D1 mRNA, accounting for the relatively low level of protein D1 synthesis observed under our experimental conditions.

The results presented (Figs. 2*C*, 4*C*, and 5) confirm that degradation of protein D1 is paralleled by the appearance of a 20-kDa C-terminal fragment (10, 17) and is therefore different from that observed after visible photoinhibition. Moreover, the observation that the time courses for the loss of photosynthetic activity and degradation of protein D1 are similar suggests that the former is the consequence of the latter rather than *vice versa*. This hypothesis seems to be compatible with the fact that ultraviolet B light at the intensity used can support only limited electron flow through the photosystems. Whether ultraviolet B-induced D1 cleavage is catalyzed by specific proteolytic activity or whether it is the direct consequence of a photolytic event is, however, still an open question. Our data also indicate that ultraviolet B-induced cleavage of protein D1 does not imply monomerization of PSII cores and that the dimeric/monomeric state of PSII is not related to the phosphorylation level of other PSII subunits. These data are clearly at variance with what is observed in the case of visible light, where degradation of protein D1 is linked with dissociation of the CP43 subunit and with lateral migration of damaged cores from granum- to stroma-exposed membranes in a phosphorylation-dependent manner (31).

Our results suggest that in the thylakoid membrane of chloroplasts treated with ultraviolet B light, degradation of proteins D1 and D2 leads to disassembling of PSII centers, leaving an excess of CP47 and CP43, which were indeed found in our sucrose gradient centrifugation experiments as free-running proteins. In contrast to what is observed after photoinhibition by visible light, no partially assembled PSII core complexes could be detected by this technique, supporting the view that full dismantling, rather than partial dissociation, of damaged centers occurs. A similar situation was described previously in cyanobacteria (32).

Fractionation of thylakoid membranes into grana, margins, and stroma-exposed lamellae indicates that there is no significant migration of damaged centers between the different compartments of the thylakoid membrane. Inspection of our electron micrographs of chloroplasts from ultraviolet B-treated plants indicates that thylakoid morphology is modified upon exposure to ultraviolet B light: thylakoids appear more vesiculated, with partial relaxation of the granum structure. This modification may lead to increased accessibility of digitonin, allowing membrane solubilization to proceed to a higher extent with an increased yield of the lighter fraction. Ultraviolet B degradation of proteins D1 and D2 may be the origin of the partial destacking of granum regions. Accordingly, data from the chlorophyll *b*-less mutant *chlorina f2*, where granum stacking is maintained essentially by PSII cores (since LHCII proteins are present only in residual amounts) (20, 43), show that the effect of ultraviolet B light on thylakoid ultrastructure is dramatic. In this case, a low number of grana with a smaller number of thylakoids per granum are found after irradiation with ultraviolet B,[2] confirming that damage to PSII can actually affect the stability of the grana. Thus, the decrease in the chlorophyll a/b ratio in stroma membranes from ultraviolet B-treated plants probably reflects partial destabilization or destacking of grana rather than actual lateral migration.

In conclusion, we have shown that ultraviolet B radiation alters the functional organization of PSII centers in a different way with respect to photoinhibitory visible light. The major difference concerns the rearrangement of the PSII structure after protein D1 degradation. In the case of ultraviolet B light, damage to the PSII reaction center does not seem to activate the series of events proposed to define the repair cycle in the case of visible light. Whether ultraviolet B light-induced damage may be repaired in higher plants by subsequent exposure to visible light, as is the case in cyanobacteria (40), will be the subject of further studies.

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Ultraviolet B Exposure of Whole Leaves of Barley Affects Structure and Functional Organization of Photosystem II

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