Membrane Protein Damage and Repair: Removal and Replacement of Inactivated 32-kilodalton Polypeptides in Chloroplast Membranes

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ABSTRACT Incubation of *Chlamydomonas reinhardii* cells at light levels that are several times more intense than those at which the cells were grown results in a loss of photosystem II function (termed photoinhibition). The loss of activity corresponded to the disappearance from the chloroplast membranes of a lysine-deficient, herbicide-binding protein of 32,000 daltons which is thought to be the apoprotein of the secondary quinone electron acceptor of photosystem II (the Q_B protein). In vivo recovery from the damage only occurred following *de novo* synthesis (replacement) of the chloroplast-encoded Q_B protein. We believe that the turnover of this protein is a normal consequence of its enzymatic function in vivo and is a physiological process that is necessary to maintain the photosynthetic integrity of the thylakoid membrane. Photoinhibition occurs when the rate of inactivation and subsequent removal exceeds the rate of resynthesis of the Q_B protein.

A 32,000-dalton integral membrane polypeptide of photosystem II (PS II)[1] is known to be the binding site for several families of herbicides, including the triazines (1). Competition studies using herbicides and quinone analogues (2) have supported the hypotheses that the herbicides act by displacement of a bound quinone (Q_B) which functions as the secondary quinone electron acceptor for PS II (3, 4). It has been accepted that the 32-kilodalton (kd) polypeptide shall be designated as the Q_B protein since it functions as the apoprotein of the bound quinone (formalized at the International Conference on Herbicides That Inhibit Photosynthesis, Wageningen, 1983; see reference 5). Pulse-labeling studies using *Spirodella* (6) and *Chlamydomonas* (7) indicate that the Q_B protein exhibits a very rapid turnover in the light.

After transfer of dark-grown maize seedlings to light, the level of the mRNA coding for the Q_B protein becomes the most abundant message in the chloroplast (8). For this reason the 32-kd protein has also been referred to as the product of a "photogene" (9). In mature leaf tissue these high mRNA levels are maintained; this corresponds to the continued high rate of synthesis (and corresponding turnover) of the Q_B protein.

The reason for the high mRNA levels and rapid turnover rate of the Q_B protein in chloroplasts has not been known. Matoo et al. (10) have suggested that the rapid turnover may in some way be related to a control mechanism for PS II function. Alternatively, Arntzen et al. (1) hypothesized that the unusually high rate of turnover of the Q_B protein in the light could be a natural consequence of its in vivo function as the secondary acceptor of PS II. (This enzymatic function involves the stabilization of reactive quinone anions in the formation of the reduced plastoquinol.) This manuscript will present data that are interpreted to support the latter idea.

Several research laboratories have shown that exposure of green plants to high photon flux densities results in a loss of photosynthetic capacity that has primarily been related to a loss of PS II function in the chloroplasts (12–14). This phenomenon is termed photoinhibition. We have recently analyzed the onset of photoinhibition in *Chlamydomonas* cells. The primary biochemical lesion was a loss of light-dependent electron flow at the level of the secondary acceptor of PS II in samples exposed to photon flux densities several times higher than those under which the cells had grown and

[1] *Abbreviations used in this paper:* kd, kilodalton; LDS, lithium dodecyl sulfate; LHC, light-harvesting chlorophyll *a*/*b*-protein complex; PS II, photosystem II.

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developed (11). This in vivo loss of activity can be mimicked in vitro by mild trypsinization of isolated thylakoids. The proteolysis affects a limited number of thylakoid surface-exposed polypeptides, including the Q_B protein (15, 16).

In the present study we have correlated the in vivo turnover of the Q_B protein to the extent of photoinhibitory damage in *Chlamydomonas* chloroplast thylakoids.

MATERIALS AND METHODS

Cell Growth and Photoinhibition: *Chlamydomonas reinhardii* cells (var. Y-1) were grown in a medium containing minerals and acetate as a carbon source in batch cultures at 25° ± 1°C under continuous illumination at 350 $\mu E/m^2$ per s as previously described (11). Photoinhibitory light was provided by a quartz-halogen lamp which generated a photon flux of 3,500 $\mu E/m^2$ per s of unfiltered white light at the level of the cells. For measurements of in vivo recovery, photoinhibited cells were washed and resuspended in fresh growth medium and further incubated at 25°C in low light (350 $\mu E/m^2$ per s) or in the dark for up to 3 h.

Preparation of Purified Thylakoid Membranes: Cell homogenates were prepared either by sonication (four times, 5 s; 40% maximum power using a model W185 sonicator, Heat Systems-Ultrasonics, Inc., Plainview, NY) or by disruption using a French pressure cell (operated at 3,000 psi at 4°C). Homogenates were layered over a discontinuous sucrose gradient (2.0, 1.75, 1.5, and 1.0 M) prepared in homogenization buffer (30 mM tricine, pH 7.8, 30 mM KCl; 2 mM $MgCl_2$) and centrifuged at 120,000 *g* for 90 min at 5°C. The membrane fraction at the interface between 1.75 and 2.0 M sucrose was collected and used either immediately or after storage at −80°C for up to 3 wk. Routine analyses of PS II-dependent electron transport using dichlorophenolindophenol ($H_2O \rightarrow$ DCPIP), or room temperature chlorophyll fluorescence inductions, were carried out as previously described (11).

Radioactive Labeling of Membrane Proteins: We assayed polypeptide synthesis or turnover by pulse-labeling intact cells with $^{35}SO_4$ (0.5–3 $\mu C/\mu mol$, 0.1 $\mu mol/ml$). Prior to labeling, cells were washed in growth medium containing only 0.01–0.05 μmol sulfate/ml and preincubated for 15 min in low light (350 $\mu E/m^2$ per s) before the addition of the radioactive sulfate. The labeling was generally carried out for 15–20 min and terminated by addition of nonradioactive sulfate (5 μmol/ml). The cells were then washed in normal growth medium and resuspended for the subsequent chase period.

Labeling experiments using [^{14}C]arginine (345 mCi/mM) and [^{14}C]lysine (10 mCi/mM) involved growth of *Chlamydomonas* cells under low light in the presence of the appropriate amino acid for 24 h during a mid-log growth phase of the cultures.

Labeling of isolated membranes with N_3-[^{14}C]atrazine (Pathfinder Laboratories, St. Louis, MO) was carried out by incubation of purified membranes at 5°C with 10 μM N_3-[^{14}C]atrazine (50 $\mu Ci/\mu mol$) for 30 min in a 10-cm open-top glass Petri dish under a germicidal UV lamp at a distance of 5 cm (2).

LDS PAGE and autoradiography were carried out as described by Kyle et al. (11).

RESULTS

Identification of the Q_B Protein in *Chlamydomonas* Thylakoids

By using [^{14}C]azidoatrazine as a specific tag of the Q_B protein (16, 17), we can identify a diffusely stained band at an approximate molecular weight of 32,000 in *Chlamydomonas* thylakoids (Fig. 1, lane *5*). This identification is further confirmed by the lack of labeling of this polypeptide using [^{14}C]lysine (lane *4*), since the Q_B protein is known to contain no lysine (6, 18–20). It does, however, contain arginine and is therefore labeled after addition of [^{14}C]arginine to the growing culture (Fig. 1, lane *3*). In addition, pulse labeling with ^{35}S for a short time (15 min) results in the tagging of several polypeptides (Fig. 1, lane *2*), including the Q_B protein at 32 kd. (The other heavily labeled proteins are the apoproteins of the light harvesting chlorophyll *a/b*-protein complex [LHC], the most abundant thylakoid proteins.) For the remainder of this manuscript, the in vivo pulse labeling of the rapidly turned over 32-kd protein by $^{35}SO_4$ will be used to identify the Q_B protein.

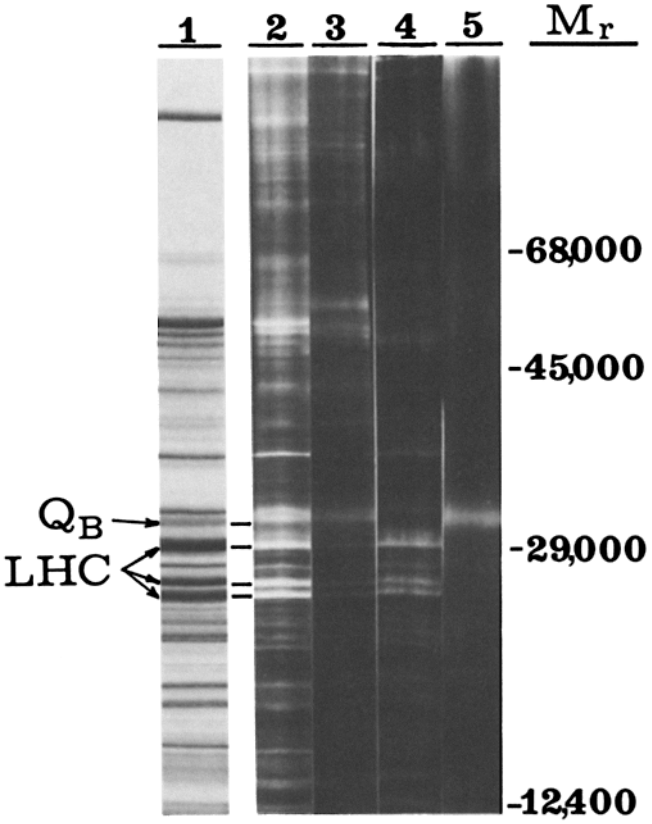

FIGURE 1 Identification of the Q_B protein in *Chlamydomonas* thylakoids. PAGE of *Chlamydomonas* thylakoids in the presence of lithium dodecyl sulfate (LDS PAGE) and 4 M urea: Lane *1*, Coomassie Blue-stained gel, lanes *2–4*, autoradiograms of the PAGE separations of *Chlamydomonas* thylakoids from cells pulse-labeled with $^{35}SO_4$ (lane *2*); or grown in [^{14}C]arginine (lane *3*) or [^{14}C]lysine (lane *4*); lane *5*, autoradiogram of *Chlamydomonas* thylakoids tagged with [^{14}C]azidoatrazine. The Q_B protein and the LHC proteins are identified.

Accelerated Turnover of the Q_B Protein in High Light

Chlamydomonas cells were pulse-labeled with $^{35}SO_4$ for 15 min and then incubated under normal growth conditions for 2 h. This latter time period was necessary to allow the integration of the newly synthesized (labeled) Q_B protein with the PS II core. The cells were then exposed for 90 min to either low or photoinhibitory (high) light intensities. The amount of Q_B protein was then analyzed by protein separation on polyacrylamide gels followed by autoradiography. Cells at the end of the $^{35}SO_4$ pulse plus 2-h incubation (Fig. 2, lane *2*) showed four major polypeptides containing label: the Q_B protein plus the three apoproteins of the LHC. Cells that were maintained in the low light (normal growth conditions) showed a small loss of Q_B protein label as compared with the LHC polypeptides (lane *3*), indicating some turnover of the Q_B protein. In contrast, cells exposed to high light showed an almost complete loss of Q_B protein with little change in the LHC polypeptides (lane *4*).

The Q_B protein is chloroplast encoded and synthesized on 70S chloroplast ribosomes (9) and its in vivo synthesis is blocked by chloramphenicol (7). We previously demonstrated that the inactivation of the Q_B protein during photoinhibition correlates directly with loss of PS II activity (11). We have now measured PS II activity via measurement of variable

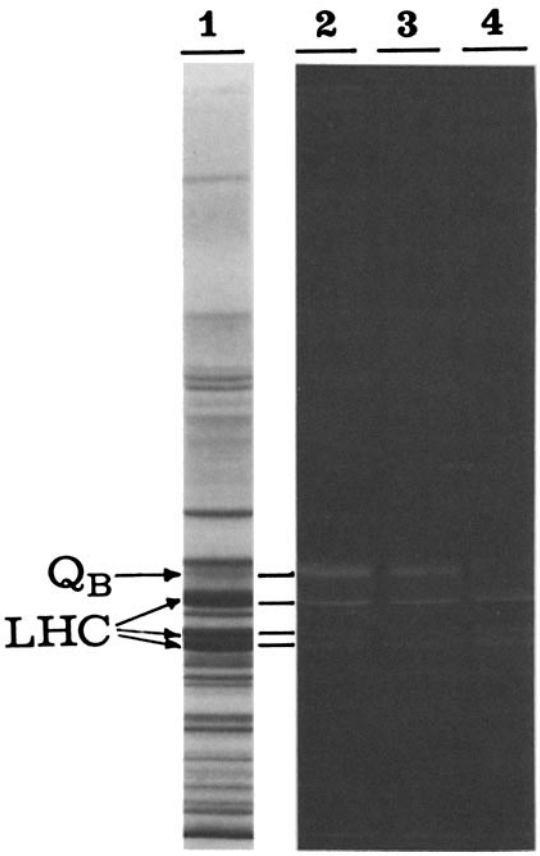

FIGURE 2 Turnover (loss from the membrane) of pulse-labeled Q_B-protein in low or high light. LDS PAGE of *Chlamydomonas* thylakoids in the presence of 4 M urea: Lane *1*, Coomassie Blue-stained gel; Lanes *2–4*, autoradiograms of the PAGE separations of thylakoids isolated from $^{35}SO_4$ pulse-labeled *Chlamydomonas* cells at zero time, i.e., prior to light treatments (lane *2*), 90-min growth under low light conditions (lane *3*), and 90 min under high light (10 times growth light intensities) (lane *4*). The LHC bands and the Q_B protein positions are noted.

fluorescence in cells in the presence or absence of chloramphenicol (Fig. 3). PS II activity declined faster and to a greater extent in the cells in the presence of chloramphenicol.

Recovery of Photodamage Requires De Novo Synthesis of the Q_B Protein

Chloroplasts of photoinhibited cells recover their PS II-dependent electron transport activity (measured by monitoring variable fluorescence) if the cells were transferred from high to low light (Fig. 4). Only partial recovery was observed in cells transferred to the dark. Recovery in low light is completely inhibited by chlorophenicol, but not by cycloheximide, indicating that chloroplast-directed protein synthesis is required for the recovery process. The slight reduction in rate of recovery in the presence of cycloheximide may suggest some involvement of cytoplasmically translated proteins for full recovery.

Cells were pulse-labeled with $^{35}SO_4$ for 60 min during the linear phase of recovery from photoinhibition (indicated in Fig. 4) to identify the polypeptides synthesized during the recovery process. The degree of recovery of electron flow as established by the variable fluorescence (Fig. 4) correlated directly with the synthesis of the Q_B protein (Fig. 5). The incorporation of the $^{35}SO_4$ into the protein occurred at the highest rate in cells incubated in light, or light with addition of cycloheximide. (Cycloheximide partially prevents $^{35}SO_4$ uptake by cells, which results in a lower specific activity of protein labeling. However, in the presence of cycloheximide, the Q_B protein acquired >90% of the total thylakoid label.) A lesser extent of Q_B protein synthesis occurred in the dark, and a scarcely detectible amount of label was observed in the Q_B protein in the light/chloroamphenicol-treated cells relative to that observed in the absence of chloramphenicol (Fig. 5).

DISCUSSION

The Q_B protein of chloroplast thylakoids functions as the apoprotein that binds secondary plastoquinone electron acceptor of PS II (1, 2, 5). As such, it is an integral component of the photosynthetic electron transport chain and its presence is a prerequisite for photosynthetic activity. We have previously reported that exposure of *Chlamydomonas reinhardii* to high photon flux densities results in a loss of photosynthetic activity (photoinhibition). This occurs as a result of blockage of electron transport at the level of the secondary acceptor of PS II (11). This manuscript presents data which we interpret as indicating that photoinhibition occurs as a result of a physical removal of the Q_B protein from the membrane, and that recovery of activity requires *de novo* synthesis (replacement) of the polypeptide.

The Q_B protein can be radioactively labeled, in vivo, by exposure of *Chlamydomonas* to a pulse of $^{35}SO_4$. We verified the fact that we were labeling the Q_B protein by the comigration of label after tagging the protein with the photoaffinity herbicide azidoatrazine, and by demonstrating the absence of lysine (but not arginine) in the protein (Fig. 1). The pulse-labeled Q_B protein was selectively lost from *Chlamydomonas* thylakoids during a 90-min exposure to photoinhibitory conditions (Fig. 2). The more rapid and extensive photoinhibition (loss of functional activity of PS II) that

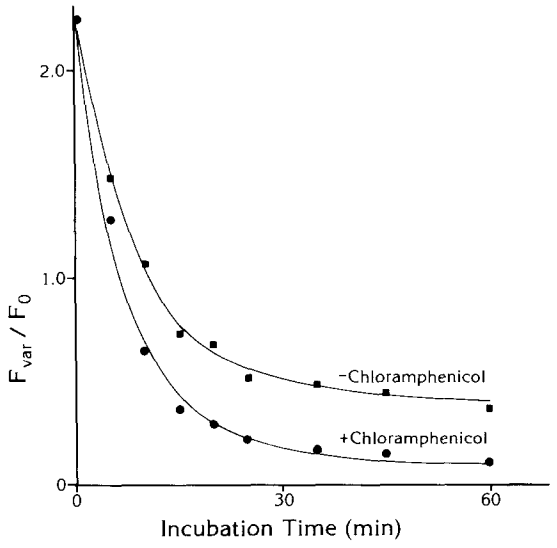

FIGURE 3 The effect of chloramphenicol during photoinhibition. Prior to incubation of *Chlamydomonas* cells in high light, chloramphenicol (200 μg/ml) was added to one of two samples. The loss of PS II function during incubation in high light was monitored as loss of the chlorophyll variable fluorescence as a function of the initial fluorescence (F_v/F_o). Fluorescence parameters were determined from in vivo transients in the presence of 10 μM diuron as described in reference 11.

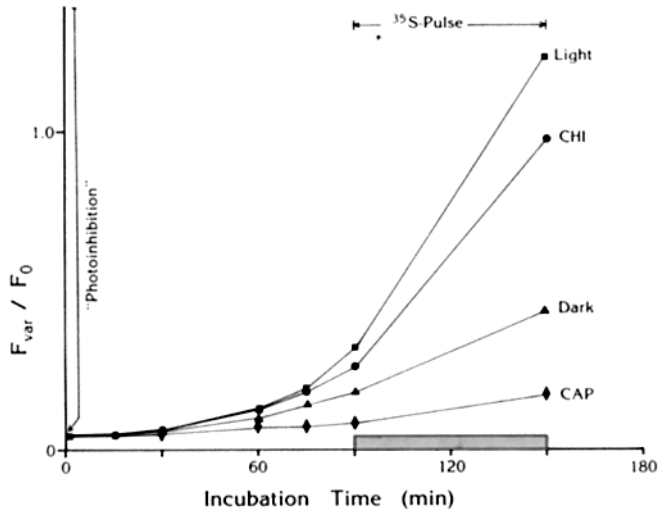

FIGURE 4 Recovery of PS II activity after photoinhibition. Variable chlorophyll fluorescence (F_v/F_o) was used as a monitor of PS II activity to measure restoration of photosynthetic capacity in *Chlamydomonas* cells that had been exposed to a 90-min photoinhibition treatment in the presence of chloramphenicol (200 μg/ml). After photoinhibiton, cells were washed five times to remove all chloramphenicol and allowed to recover under normal growth light conditions with no further additions (■), in the presence of cycloheximide (*CHI*; 2 μg/ml) (●), or in the presence of chloramphenicol (*CAP*; 200 μg/ml) (◆). One sample was kept in the dark during the recovery period (▲). Variable fluorescence values plotted are the means of four measurements (SE ± 5%) of a single experiment. Other experiments yielded qualitatively similar results but the overall fluorescence values were dependent on the physiology of the cells and the extent of photoinhibition. Pulse labeling with $^{35}SO_4$ during the recovery was carried out between 90 and 150 min after the termination of the photoinhibition.

occurred in the presence of chloramphenicol (Fig. 3) can be interpreted as indicating that there is a normal, ongoing rate of synthesis of the Q_B protein which serves to repair, and thereby partially prevent, the loss of PS II function.

The observation that chloroamphenicol but not cycloheximide blocked both recovery from photoinhibition and the parallel Q_B protein synthesis (Figs. 4 and 5) indicates that it is specifically the loss of the Q_B protein function that must be "repaired" to regain photosynthetic competence in photoinhibited *Chlamydomonas*. The light requirement for this recovery suggests either a light-regulated step in protein synthesis, a requirement for photosynthetic activity (such as ATP from cyclic photophosphorylation) for protein synthesis, or a possible energized membrane requirement for the insertion and integration of the Q_B protein into the PS II complex of thylakoids.

The Q_B protein is synthesized at rates equivalent to the most abundant proteins of the chloroplast, yet it remains a minor membrane constituent. Its rate of turnover has been estimated (10, 20) to be 50 to 60 times more rapid than the LHC protein (the most abundant thylakoid polypeptide). There has been no obvious explanation for this rapid turnover other than the suggestion that the protein plays a regulatory role as a "protein shield" which influences PS II electron transport (10). We can now be more specific in assigning a reason for the relatively rapid turnover rate: the protein is damaged as a consequence of its function as a quinone-binding polypeptide.

The electron transfer reactions of PS II require that Q_B functions as a two-electron "gate" (21). Single electrons arrive from the reaction-center chlorophyll and the Q_B protein stabilizes the singly reduced quinone anion (Q_B^-), which allows the formation of the fully reduced quinone (Q_B^{2-} or Q_BH_2) in a two-step process. Once reduced, the plastohydroquinone is released from its binding site in the Q_B protein and exchanges for an oxidized quinone in the lipid-soluble membrane "quinone pool." This allows the reduced quinone to diffuse to its site of oxidation, providing electrons for photosystem I. We have documented that photoinhibitory conditions result in the loss of electron transport in *Chlamydomonas* thylakoids at the level of Q_B (11). We believe this results from the interaction of molecular oxygen with a quinone anion, thus generating oxygen radicals directly within the quinone binding site of the Q_B protein, and thereby causing a direct chemical alteration of the Q_B protein (discussed in more detail in reference 11).

Our present study has shown that the Q_B protein is turned over when the Q_B function is lost due to photoinhibition. We can now propose that chemical modification of the Q_B protein (as described above) elicits the proteolytic degredation of the damaged electron carrier. Thus, *de novo* synthesis of the protein is required to recover electron transport activity.

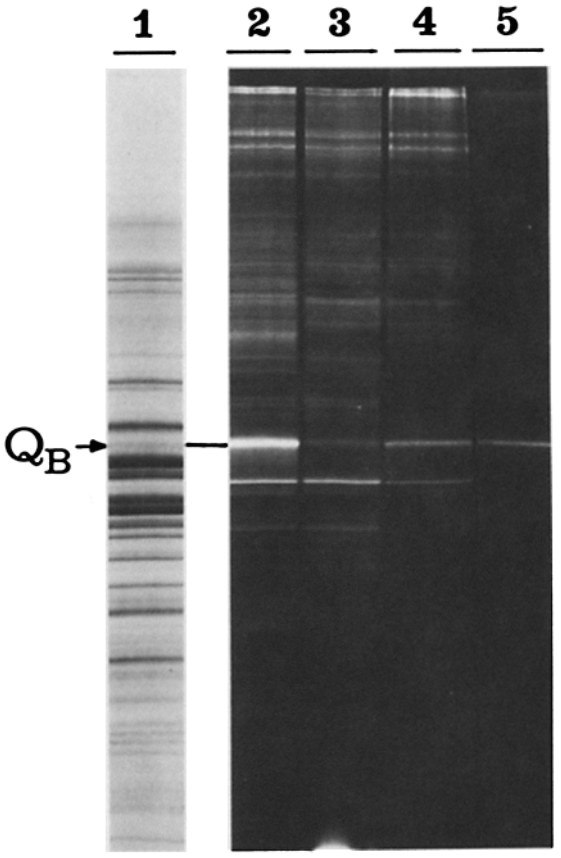

FIGURE 5 Protein synthesis during the recovery from photoinhibition. Lane *1* is the Coomassie Blue-stained polypeptide profile of *Chlamydomonas* thylakoids separated by LDS PAGE in the presence of 4 M urea. Lanes *2–5* are audoradiograms from LDS PAGE gels of *Chlamydomonas* thylakoids (10 μg chlorophyll loaded per lane) pulse-labeled during recovery from photoinhibition as indicated in Fig. 4. Lane *2*, recovery in the light; lane *3*, recovery in light in the presence of chloramphenicol; lane *4*, recovery in the dark; and lane *5*, the recovery in the light in the presence of cycloheximide. The Q_B protein is identified with an arrow. Further experimental details are in the legend to Fig. 4.

Obviously, the formation of quinone anions and the presence of oxygen are obligate steps in the photosynthetic process of all higher plant chloroplasts. It is therefore not surprising that there is rapid turnover of the 32,000-dalton Q_B protein in all higher plants thus far examined (22). As such, an understanding of the turnover events will aid in our understanding of how primary photosynthetic activity is maintained under field conditions. In addition, the fact that photoinhibition can provide such a highly targeted site of membrane protein damage indicates that this phenomenon can be used as a simple way to induce defined damage sites in membranes. These damaged membranes can be used as models to study the mechanism(s) by which damaged membrane proteins are recognized, selectively removed, and subsequently replaced.

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REFERENCES

1. Arntzen, C. J., K. E. Steinback, W. F. J. Vermaas, and I. Ohad. 1983. Molecular characterization of the target site(s) for herbicides which affect photosynthetic electron transport. *In* Pesticide Chemistry: Human Welfare and the Environment, Vol. 3. S. Matusunka, D. H. Hutson, and S. D. Murphy, editors. Pergammon Press, Oxford. 51–58.
2. Vermaas, W. F. J., C. J. Arntzen, L.-Q. Gu, and C.-A. Yu. 1983. Interactions of herbicides and azidoquinones at a photosystem II binding site in the thylakoid membrane. *Biochem. Biophys. Acta.* 723:266–275.
3. Velthuys, B. R. 1981. Electron-dependent competition between plastoquinone and inhibitors for binding to photosystem II. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 126:277–281.
4. Wraight, C. A. 1981. Oxidation-reduction physical chemistry of the acceptor complex in bacterial photosynthetic reaction centers: evidence for a new model of herbicide action. *Isr. J. Chem.* 21:348–354.
5. Hirschberg, J. A. Bleecker, D. J. Kyle, L. McIntosh, and C. J. Arntzen. 1984. The molecular basis of traizine-herbicide resistance in higher plant chloroplasts. *Z. Naturforsch C.* In press.
6. Edelman, M., and A. Reisfeld. 1978. Characterization, translocation and control of the 32,000 dalton chloroplast membrane protein in *Spirodella. In* Chloroplast Development. G. Akoyunoglou and J. H. Argyroudi-Akoyunoglou, editors. Elsevier/North Holland, Amsterdam. 641–652.
7. Wettern, M., G. C. Owens, and I. Ohad. 1983. Role of thylakoid polypeptide phosphorylation and turnover in the assembly and function of photosystem II. *Methods Enzymol.* 97:554–567.
8. Bedbrook, J. R., G. Link, D. M. Coen, L. Bogorad, and A. Rich. 1978. Maize plastid gene expressed during photoregulated development. *Proc. Natl. Acad. Sci. USA.* 75: 3000–3064.
9. Bogorad, L. 1981. Chloroplasts. *J. Cell Biol.* 91 (3, Pt. 2):256s–270s.
10. Matoo, A. K., U. Pick, H. Hoffman-Falk, and M. Edelman. 1981. The rapidly metabolized 32,000 dalton polypeptide of the chloroplast is the "proteinaeous shield" regulating photosystem II electron transport and mediating diuron sensitivity. *Proc. Natl. Acad. Sci. USA.* 78:1572–1576.
11. Kyle, D. J., I. Ohad, and C. J. Arntzen. 1983. Membrane protein damage and repair: selective loss of a quinone protein function in chloroplast membranes. *Proc. Natl. Acad. Sci. USA.* In press.
12. Powles, S. 1984. Photoinhibition of photosynthesis induced by visible light. *Annu. Rev. Plant Physiol.* 35:15–44.
13. Osmond, C. B. 1981. Photorespiration and photoinhibition: some implications for the energetics of photosynthesis. *Biochim. Biophys. Acta.* 639:77–98.
14. Bjorkman, O. 1981. Responses to different quantum flux densities. *In* Physiological Plant Ecology, Vol. I. O. L. Lange, P. S. Nobel, C. B. Osmond, and H. Ziegler, editors. Springer-Verlag, Berlin. 57–108.
15. Renger, G., R. Hagemann, and G. Dohnt. 1983. The role of lysine residues for excitation energy transfer and herbicide action in system II. *Photobiochem. Photobiophys.* 5:273–273.
16. Steinback, K. E., L. McIntosh, L. Bogorad, and C. J. Arntzen. 1981. Identification of the trizine receptor protein as a chloroplast gene product. *Proc. Natl. Acad. Sci. USA.* 78:7463–7467.
17. Pfister, K., K. E. Steinback, G. Gardner, and C. J. Arntzen. 1981. Photoaffinity labelling of an herbicide receptor protein in chloroplast membranes. *Proc. Natl. Acad. Sci. USA.* 78:981–985.
18. Zurawski, G., H. J. Bohnert, P. R. Whitfeld, and W. Bottomley. 1982. Nucleotide sequence of the gene for the 32,000-M_r thylakoid membrane protein from *Spinacia oleracea* and *Nicotiana debneyi* predicts a totally conserved primary translation product of M_r 38,950. *Proc. Natl. Acad. Sci. USA.* 79:7699–7703.
19. Hirschberg, J., and L. McIntosh. 1983. Molecular basis of herbicide resistance in *Amaranthus hybridus. Science (Wash. DC).* 222:1346–1348.
20. Edelman, M., A. K. Matoo, and J. B. Marder. 1983. Three hats of the rapidly-metabolized 32 kilodalton protein of the thylakoids. *In* Chloroplast Biogenesis. R. J. Ellis, editor. Cambridge University Press, Cambridge, UK. In press.
21. Crofts, A. R., and C. A. Wraight. 1983. The electrochemical domain of photosynthesis. *Biochim. Biophys. Acta.* 726:149–185.
22. Hoffman-Falk, H., A. K. Matoo, J. B. Marder, M. Edelman, and R. J. Ellis. 1982. General occurrance and structural similarity of the rapidly synthesized, 32,000 dalton protein of the chloroplast membrane. *J. Biol. Chem.* 257:4583–4587.