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Photoinduced degradation of the D1 polypeptide in isolated reaction centers of photosystem II: Evidence for an autoproteolytic process triggered by the oxidizing side of the photosystem

(protein turnover/photoinhibition)

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ABSTRACT **When the isolated D1/D2/cytochrome b_{559} complex was exposed to bright light, a distinctive pattern of D1 polypeptide fragments was observed under both aerobic and anaerobic conditions. The major degradation product had an apparent molecular mass of 24 kDa, while other fragments were detected at 17, 14, and 10 kDa by immunoblotting. This pattern was observed when the electron acceptors 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone or silicomolybdate were present during illumination. It is known that these conditions stabilize $P680^+$ chlorophyll and bring about the photooxidation and destruction of pigments in the reaction center, particularly chlorophyll absorbing at 670 nm and β-carotene. When $P680^+$ was not allowed to accumulate, either by omission of an electron acceptor or by addition of both an electron donor (Mn^{2+}) and an acceptor, no breakdown fragments were observed. In the former case, however, some degradation of the D1 and D2 polypeptides did occur. Under conditions that gave rise to the characteristic D1 breakdown pattern, the D2 polypeptide was also degraded to specific fragments detected at about 29 and 21 kDa by immunoblotting. The results indicate that the photoinduced degradation of D1 (and D2) does not involve exogenous proteases but is most likely an autoproteolytic process. Moreover, our data indicate that the photochemical damage giving rise to D1 and D2 degradation occurs on the oxidizing rather than the reducing side of photosystem II and involves photooxidation of the accessory pigments. The results are discussed in terms of D1 and D2 turnover and photoinhibition.**

The rapid turnover of the 32-kDa D1 polypeptide (1) of the reaction center of photosystem II (PSII) is one of the most intriguing phenomena of oxygenic photosynthesis, especially since this protein binds key components involved in primary charge separation and oxygen evolution. During the past few years, evidence has accumulated that D1 turnover is a crucial part of a repair system required because of an inherent vulnerability of the PSII reaction center to damage by light (2, 3). It is now generally believed that the extent of photoinhibition, bringing about a loss of photosynthetic efficiency observed *in vivo*, results from the balance between the rate of photodamage to PSII and the rate of its repair (3). Under optimal conditions the rate of photodamage does not exceed the rate of repair, and therefore no photoinhibition is observed. Under adverse conditions (e.g., strong light and low ambient temperatures), the repair process does not keep pace with the rate of damage and photoinhibition occurs.

The details of the photochemical processes that induce the damage, and the nature of the triggering mechanisms that bring about D1 turnover, are unknown. The primary cleavage site on the D1 polypeptide is thought to be close to an α-helix-destabilizing stretch of amino acids rich in glutamate, serine, and threonine residues ("EST" region; ref. 4). Such regions are believed to signal rapid degradation of proteins in eukaryotes (5). In the case of the D1 polypeptide, an initial breakdown fragment of 23.5 kDa has been observed (4, 6). Proteolytic mapping (6) has suggested that this fragment may be derived from a primary cleavage at a site adjacent to the EST region within a motif consisting of Gln-Glu-Glu-Glu-Thr (residues 241–245), while N-terminal sequencing of an 8-kDa breakdown fragment has indicated that the cleavage may occur closer to Arg-238, possibly at Phe-239 (7).

Until recently it was thought that the protease responsible for this cleavage was not active *in vitro* (8). However, a characteristic pattern of D1 breakdown products was detected when isolated thylakoids (9, 10) and oxygen-evolving PSII core preparations (11) were exposed to high light intensities. From these studies, particularly the latter, it was concluded that the protease responsible for the photoinduced degradation of the D1 polypeptide was located in a stoichiometric amount within the PSII core complex. In this paper, we show that a similar photoinduced breakdown pattern occurs in a much simpler system—namely, the isolated PSII reaction center consisting of the D1 and D2 polypeptides, the α and β subunits of cytochrome b_{559}, and the product of the *psbI* gene. Our results, therefore, place serious doubts on the existence of a specific protease involved in D1 degradation and indicate that the generation of the observed pattern of photoinduced breakdown products is due to an autoproteolytic mechanism. Also our data indicate that the photochemical damage giving rise to D1 degradation occurs on the oxidizing rather than the reducing side of the PSII reaction center. In addition we observe photoinduced breakdown fragments of the related D2 polypeptide.

MATERIALS AND METHODS

The reaction centers of PSII were isolated from peas by a modification (12) of the procedure of Nanba and Satoh (13).

Light treatments were performed in a stirred glass cuvette maintained at 20°C for 30 min, with a chlorophyll concentration of 50 $\mu g \cdot ml^{-1}$ in 50 mM Tris/2 mM dodecyl β-D-maltoside, pH 8.0. Heat-filtered (Schott filter KG1) white light [4 $mE \cdot m^{-2} \cdot s^{-1}$; 1 E (einstein) = 1 mol of photons] was produced by an incandescent Flexilux 650 lamp. The electron acceptors 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB, a gift from W. Oettmeier, Ruhr-Universitaet Bochum, Bochum, F.R.G.) and silicomolybdate (obtained from Pfaltz & Bauer) were used at 200 and 250 μM, respectively. Mn^{2+} was used as an electron donor at 10 mM concentration. Anaerobic conditions were achieved as re-

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Abbreviations: PSII, photosystem II; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone.

quired by the addition of glucose (5 mM), catalase (0.1 mg·ml^{-1}), and glucose oxidase (0.1 mg·ml^{-1}) and the cuvette was repeatedly flushed with nitrogen prior to illumination.

Gradient gels (10–17% polyacrylamide) containing 6 M urea were used for analysis of the polypeptide composition of samples. Gels were either stained with Coomassie blue for protein visualization or prepared for Western immunoblotting (14). Profiles of separated proteins were electrophoretically transferred onto nitrocellulose (14) and detected using rabbit primary antibodies to the D1 and D2 proteins generated by the expression of wheat *psbA* and *psbD* genes in *Escherichia coli* (15). Alkaline phosphatase-conjugated secondary antibodies (Sigma) were then employed, followed directly by the appropriate chromogenic substrates.

RESULTS

Fig. 1 shows the results of an experiment in which the isolated PSII reaction centers were illuminated by intense white light for 30 min in the presence of the quinone DBMIB. Fig. 1*a* is a Coomassie blue-stained SDS/polyacrylamide gel showing that the illumination treatment caused a significant degree of degradation of the D1 and D2 polypeptides relative to the 9-kDa α subunit of cytochrome b_{559}. Coomassie blue staining did not reveal any degradation products of these polypeptides. However, a more sensitive immunological method, using an antibody to the *psbA* gene product (15), indicated an interesting pattern of breakdown products dominated by a 24-kDa fragment (Fig. 1 *b* and *c*). The overdeveloped Western blot (Fig. 1*c*) revealed other breakdown products at 17, 14, and 10 kDa. None of these fragments or the degradation of any polypeptides could be detected after the control treatments; lane 0 shows a sample taken for solubilization immediately after dilution into treatment buffer containing DBMIB, and lane D shows a similar sample incubated in darkness for 30 min prior to solubilization. Very similar results were obtained when the same experiment was conducted under anaerobic conditions except that the time course for the appearance of the breakdown products was slightly slower (data not shown). DBMIB binds to the isolated reaction center, substituting for the lost endogenous quinones Q_A and Q_B (16). As a consequence, illumination of the reaction center reconstituted with DBMIB gives rise to the photooxidation of P680 chlorophyll as observed by flash absorption spectroscopy (16). It has been found that silicomolybdate can also act as an effective electron acceptor with the isolated PSII reaction center (17–19). Exposure of the reaction center complex to bright light in the presence of silicomolybdate under anaerobic conditions gave rise to photoinduced breakdown products (Fig. 2) similar to those produced when DBMIB was present. A similar result was obtained under aerobic conditions.

In contrast, no fragments of the D1 polypeptide were observed after illumination of the reaction centers in the absence of an electron acceptor (Fig. 3). Nevertheless, under these conditions there was a partial loss of the D1 polypeptide. Also, after photodamage in the absence of acceptor the D1 polypeptide ran at a higher molecular mass on SDS/PAGE (Fig. 3), an observation also noted in the presence of an electron acceptor (Figs. 1 and 2). When isolated reaction centers are illuminated in the presence of an electron acceptor (silicomolybdate) plus a donor (Mn^{2+}), net electron flow is catalyzed from Mn^{2+} to silicomolybdate (20). Under such conditions, virtually no loss or photomodification of the D1 polypeptide occurred (Fig. 4).

Of further interest is the breakdown pattern observed for the D2 polypeptide when the illumination was performed in the presence of the electron acceptor DBMIB (Fig. 5). These breakdown fragments had apparent molecular masses of 29 and 21 kDa and were detected by Western blotting with an antibody raised to the *psbD* gene product (15). The D2 Western blot shown in Fig. 5 also shows the higher molecular mass band (around 60 kDa) which has been attributed to a D1/D2 heterodimer (14). In contrast, the D2 antibody did not react with a 39-kDa band detected by immunoblotting with D1 antiserum that can clearly be seen in Figs. 1–4. The origin of this band is unclear and its appearance was unpredictable.

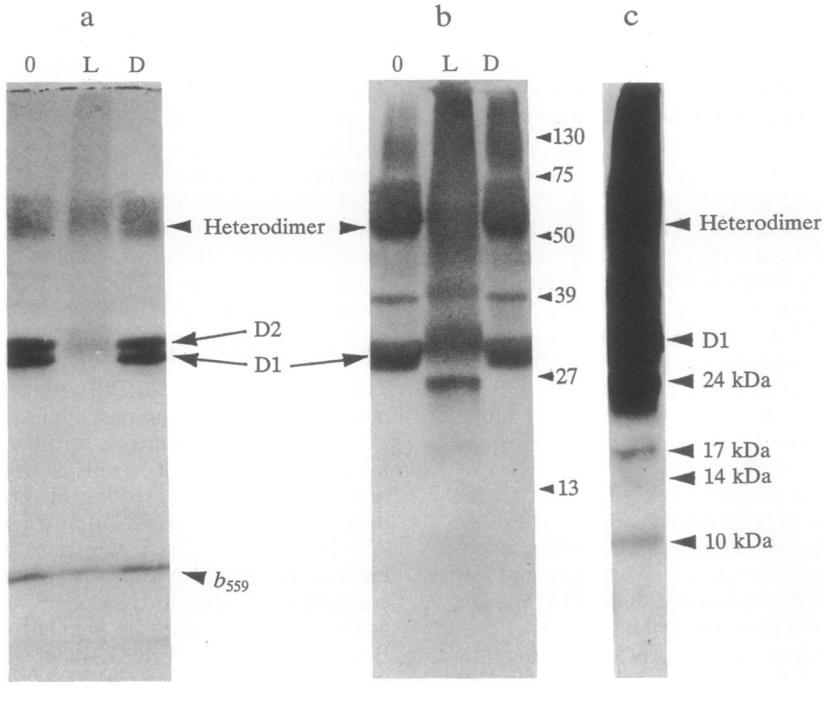

FIG. 1. (*a*) Coomassie blue-stained SDS/PAGE profiles from isolated PSII reaction centers diluted into buffer containing 200 μM DBMIB. Samples were taken for solubilization directly after dilution (lane 0), after 30 min of light treatment (lane L), or after 30 min of incubation in darkness (lane D). All samples were maintained at 20°C throughout the experiment. (*b*) Immunoblot analysis of the same profiles with D1 antiserum. Positions of molecular mass (kDa) standards are indicated. (*c*) Overdevelopment of lane L of *b* to enable visualization of fainter D1 antiserum-reactive bands produced during the light treatment.

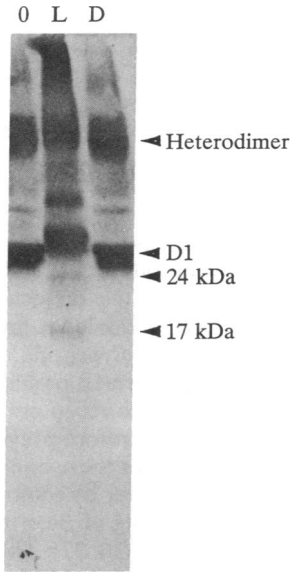

FIG. 2. Immunoblot, with D1 antiserum, of PSII reaction centers treated in the presence of 250 μM silicomolybdate under anaerobic conditions. Lanes: 0, sample taken immediately after dilution; L, after 30 min of light; D, after 30 min of darkness. All treatments were performed at 20°C and were followed immediately by solubilization.

However, it did seem to increase in intensity after the illumination period, whether or not various additions to the reaction center had been made. Also under conditions when photodamage occurred, the immunoblot assays of D1 and D2 consistently showed general smearing at higher molecular masses, the reason for which is unknown. Like D1, the unfragmented D2 polypeptide migrated at a slightly higher apparent molecular mass after treatment by illumination.

DISCUSSION

Our results show that the characteristic photoinduced breakdown of the D1 polypeptide observed in thylakoid membranes (9, 10) and oxygen-evolving PSII core preparations (11) can also be found when the isolated PSII reaction center

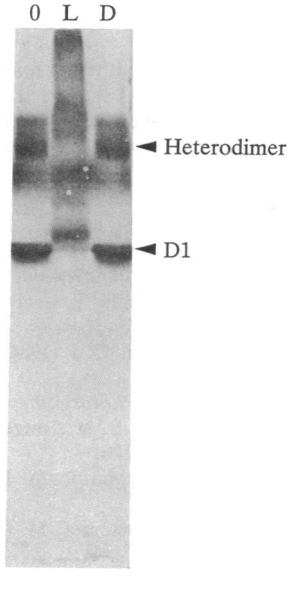

FIG. 3. Immunoblot, with D1 antiserum, of PSII reaction centers diluted into buffer containing no additions (no electron acceptor). One sample was removed (lane 0) and solubilized immediately. The remainder was incubated for 30 min at 20°C either under illumination (lane L) or in the dark (lane D) prior to solubilization.

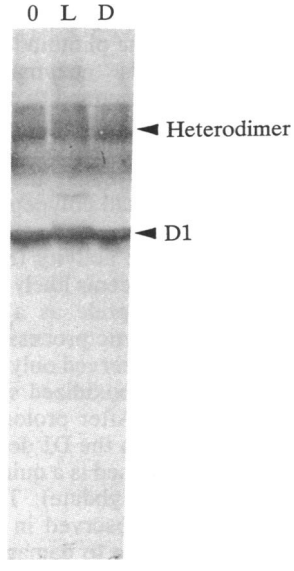

FIG. 4. Immunoblot, with D1 antiserum, of PSII reaction centers diluted into buffer containing 250 μM silicomolybdate and 10 mM $MnCl_2$. Samples were solubilized for SDS/PAGE at time zero (lane 0) or after 30 min incubation in the light (lane L) or in darkness (lane D) at 20°C.

is illuminated under conditions where an electron acceptor is present and the state $P680^+$ is able to photoaccumulate. It also seems likely, but not yet proven, that the breakdown product of about 24 kDa observed in all these studies is the same as the 23.5-kDa D1 breakdown product reported by Greenberg *et al.* (4) in their experiments with intact *Spirodela oligorrhiza*. Those workers (4) and Shipton *et al.* (6) gave evidence to support the contention that this 23.5-kDa product was an N-terminal fragment of the D1 polypeptide, but neither group detected the C-terminal fragment. However, Greenberg *et al.* (4) did report that they observed possible breakdown fragments of 8 to 14 kDa, and Trebst and Depka (7) detected an 8-kDa fragment. The relationship of these lower molecular mass fragments to those reported here and in refs. 9–11 is unclear.

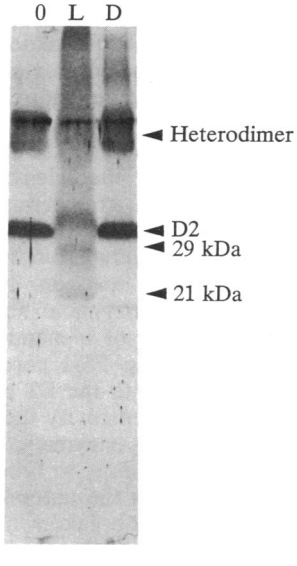

FIG. 5. Immunoblot, with D2 antiserum, of PSII reaction centers diluted into buffer containing 200 μM DBMIB. Samples were removed and solubilized immediately after dilution (lane 0) or after 30 min of incubation under light (lane L) or in darkness (lane D) at 20°C.

There are four very important implications of our findings. (*i*) It now seems unlikely that the photoinduced degradation of the D1 polypeptide *in vivo* is "enzymatic," involving a specific protease, as has been assumed to date. The isolated reaction center contains only the α and β subunits of cytochrome b_{559} and a 4.8-kDa polypeptide [the product of the *psbI* gene (21, 22)] in addition to the D1 and D2 proteins (14). Although the function of the small polypeptide produced by the *psbI* gene is unknown, it has no feature in its primary structure indicative of protease activity (Steven Hall, personal communication). Thus it seems likely that the selective degradation of the D1 polypeptide as a consequence of photodamage is an autoproteolytic process. (*ii*) The characteristic breakdown pattern is observed only under conditions in which $P680^+$ and other photooxidized species are generated in the isolated complex. After prolonged illumination there is no obvious difference in the D1 degradation pattern whether the electron acceptor used is a quinone (DBMIB) or an inorganic species (silicomolybdate). This supports the concept that photoinhibition observed in intact photosynthetic tissue is not primarily due to damage induced by the reduction of plastoquinone in the Q_B site, as has often been suggested (3, 23, 24), but rather is a consequence of the production of highly oxidizing species on the donor side of PSII as suggested by others (25–27). (*iii*) The same breakdown pattern is produced whether oxygen is present or not. This rules out the possibility that oxygen radicals are involved in D1 degradation. The same conclusion was recently reported by Jegerschöld and Styring (28). However, in the case of DBMIB there is an oxygen effect, which can be understood in terms of a quinone-dependent cyclic electron flow around PSII involving cytochrome b_{559}. When the isolated reaction center is reconstituted with quinones, the action of light is to bring about a photoreduction of cytochrome b_{559} (12, 16, 29). The reduced cytochrome is then oxidized directly or indirectly by $P680^+$ (30). This cycle thus prevents the photoaccumulation of oxidized species on the donor side of the isolated PSII reaction center. This protective mechanism, which has also been proposed by Thompson and Brudvig (31), is less efficient when oxygen is present, since it can compete as an electron acceptor. No such protective cycle operates when silicomolybdate is used as an electron donor. These differences affect the rate at which the breakdown products appear without changing the overall pattern (unpublished data). (*iv*) Under conditions where D1 is degraded, so is D2, and the breakdown products are of a different size. The photoinduced breakdown of D2 into smaller fragments has been reported previously (11), but in general it seems that D2 is more resistant to breakdown than D1 in intact tissue (32).

Aro *et al.* (9) have demonstrated that the breakdown of the D1 protein is not due to a direct photocleavage event. They showed that the primary effect is a temperature-independent photochemical damage and that this gives rise to a secondary process that brings about D1 degradation in a temperature-sensitive and light-independent reaction. They interpreted their results to indicate the enzymatic nature of the D1 degradation and the existence of a membrane-associated protease that is present in the PSII core complex in a stoichiometric amount relative to the D1 polypeptide. We suggest that the temperature sensitivity they observed and interpreted as evidence of an enzymatic process simply reflects the need for thermal energy to bring about conformational changes that result in the autoproteolytic breakdown of D1. Presumably the D2 breakdown is also autoproteolytic.

Our results place importance on the use of the isolated PSII reaction center to investigate the molecular mechanisms that underlie the physiological phenomenon of photoinhibition observed in higher plants, algae, and cyanobacteria (33). When the isolated PSII reaction center is illuminated in the absence of an acceptor, the radical pair $P680^+Pheo^-$ (where Pheo is pheophytin) is formed, which then recombines and generates the P680 triplet state (34, 35). Under these conditions there is an oxygen-dependent, preferential photodestruction of chlorophyll that absorbs at 680 nm (36). It was demonstrated that oxygen quenches the P680 triplet and is converted to its singlet state (35). The very reactive singlet oxygen then selectively attacks the P680 chlorophylls. Since in this case no breakdown products were observed, it seems unlikely that this reaction is the molecular basis for the formation of the 23.5-kDa breakdown product associated with the turnover of the D1 polypeptide. However, in the presence of acceptors (e.g., DBMIB or silicomolybdate) the situation is quite different. These acceptors compete with the recombination reaction and therefore prevent or reduce the formation of P680 triplet. Under these conditions the photodestruction of the reaction center by prolonged illumination involves a preferential bleaching of β-carotene and of chlorophylls that absorb at 670 nm (36, 37). This photobleaching is mediated by the stabilization of the highly oxidizing species $P680^+$. It is under these conditions that we observe the characteristic D1 polypeptide breakdown pattern. As shown in Fig. 4, when $P680^+$ is not allowed to photoaccumulate, by introduction of Mn^{2+} as an electron donor, no such pattern appears.

In summary, we conclude that the photoinduced degradation of the D1 polypeptide to specific breakdown products is probably an autoproteolytic process and does not involve a specific protease as was previously thought. (We cannot, however, totally dismiss the possibility of a contaminating protease at low levels that has the capacity to hydrolyze the D1 and D2 polypeptides under specific conditions.) We conclude that the molecular basis of this degradation is the stabilization of $P680^+$ and the photooxidation and destruction of accessory pigments. It is important to note that the degradation of D1 to its characteristic breakdown products occurs under anaerobic conditions, indicating that oxygen radicals are not involved in this process. Further, we have also detected a characteristic pattern for a photoinduced degradation of the D2 polypeptide that is different from that observed for D1.

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1. Edelman, M., Goloubinoff, P., Marder, J. B., Fromm, H., Devic, M., Fluhs, R. & Mattoo, A. K. (1985) in *Molecular Form and Function of the Plant Genome*, eds. van Vloten-Doting, L., Groot, G. S. P. & Hall, T. C. (Plenum, New York), pp. 291–300.
2. Kyle, D. J., Ohad, I. & Arntzen, C. J. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 4070–4074.
3. Kyle, D. J. (1987) in *Topics in Photosynthesis: Photoinhibition*, eds. Kyle, D. J., Osmond, C. B. & Arntzen, C. J. (Elsevier, Amsterdam), Vol. 9, pp. 197–226.
4. Greenberg, B. M., Gaba, V., Mattoo, A. K. & Edelman, M. (1987) *EMBO J.* **6**, 2865–2869.
5. Rogers, S., Wells, R. & Rechsteiner, M. (1986) *Science* **234**, 364–368.
6. Shipton, C. A., Marder, J. B. & Barber, J. (1990) *Z. Naturforsch. C: Biochem. Biophys. Biol. Virol.* **45**, 388–394.
7. Trebst, A. & Depka, B. (1990) *Z. Naturforsch. C: Biochem. Biophys. Biol. Virol.* **45**, 765–771.
8. Schuster, G., Shochat, S., Adir, N. & Ohad, I. (1989) in *Techniques and New Developments in Photosynthesis Re-*

search, eds. Barber, J. & Malkin, R. (Plenum, New York), pp. 499–510.
9. Aro, E.-M., Hundal, T., Carlsberg, I. & Andersson, B. (1990) *Biochim. Biophys. Acta* **1019**, 269–275.
10. Wettern, M. & Galling, G. (1985) *Planta* **166**, 474–482.
11. Virgin, I., Ghanotakis, D. F. & Andersson, B. (1990) *FEBS Lett.* **269**, 45–48.
12. Chapman, D. J., Gounaris, K. & Barber, J. (1990) in *Methods in Plant Biochemistry*, ed. Rogers, L. J. (Academic Press, London), Vol. 5, pp. 171–193.
13. Nanba, O. & Satoh, K. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 109–112.
14. Marder, J. B., Chapman, D. J., Telfer, A., Nixon, P. J. & Barber, J. (1987) *Plant Mol. Biol.* **9**, 325–333.
15. Nixon, P. J., Dyer, T. A., Barber, J. & Hunter, C. N. (1987) *FEBS Lett.* **209**, 83–86.
16. Satoh, K., Hansson, Ö. & Mathis, P. (1990) *Biochim. Biophys. Acta* **1016**, 121–126.
17. Barber, J., Chapman, D. J. & Telfer, A. (1987) *FEBS Lett.* **220**, 67–73.
18. Telfer, A., Durrant, J. & Barber, J. (1990) *Biochim. Biophys. Acta* **1018**, 168–172.
19. Takahashi, Y., Satoh, K. & Itoh, S. (1989) *FEBS Lett.* **255**, 133–138.
20. Chapman, D. J., Gounaris, K. & Barber, J. (1988) *Biochim. Biophys. Acta* **933**, 423–431.
21. Ikeuchi, M. & Inoue, Y. (1988) *FEBS Lett.* **241**, 99–104.
22. Webber, A. N., Packman, L., Chapman, D. J., Barber, J. & Gray, J. C. (1989) *FEBS Lett.* **242**, 259–262.
23. Ohad, I., Koike, H. K., Shochat, S. & Inoue, Y. (1988) *Biochim. Biophys. Acta* **933**, 288–298.
24. Greenberg, B. M., Gaba, V., Canaani, O., Malkin, S. & Mattoo, A. K. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6617–6620.
25. Cleland, R. E., Melis, A. & Neale, P. J. (1986) *Photosynth. Res.* **9**, 79–88.
26. Demeter, S., Neale, P. J. & Melis, A. (1987) *FEBS Lett.* **214**, 370–374.
27. Styring, S., Virgin, I., Ehrenberg, A. & Andersson, B. (1990) *Biochim. Biophys. Acta* **1015**, 269–278.
28. Jegerschöld, C. & Styring, S. (1991) *FEBS Lett.* **280**, 87–90.
29. Gounaris, K., Chapman, D. J. & Barber, J. (1988) *FEBS Lett.* **240**, 143–147.
30. Mathis, P., Satoh, K. & Hansson, Ö. (1989) *FEBS Lett.* **251**, 241–244.
31. Thompson, L. K. & Brudvig, G. W. (1988) *Biochemistry* **27**, 6653–6658.
32. Schuster, G., Timbert, R. & Ohad, I. (1988) *Eur. J. Biochem.* **177**, 403–410.
33. Powles, S. (1984) *Annu. Rev. Plant Physiol.* **35**, 15–44.
34. Takahashi, Y., Hansson, Ö., Mathis, P. & Satoh, K. (1987) *Biochim. Biophys. Acta* **893**, 49–59.
35. Durrant, J. R., Giorgi, L. B., Barber, J., Klug, D. R. & Porter, G. (1990) *Biochim. Biophys. Acta* **1017**, 167–175.
36. Telfer, A., He, W.-Z. & Barber, J. (1990) *Biochim. Biophys. Acta* **1017**, 143–151.
37. Telfer, A., de La Rivas, J. & Barber, J. (1991) *Biochim. Biophys. Acta*, in press.