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Breakdown of the Photosystem II Reaction Center D1 Protein under Photoinhibitory Conditions: Identification and Localization of the C-Terminal Degradation Products

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ABSTRACT: Illumination of a suspension of thylakoids with light at high intensity causes inhibition of the photosystem II electron transport activity and loss from the membrane of the D1 protein of the photosystem II reaction center. Impairment of the electron transport activity and depletion of D1 protein from the thylakoid membrane of pea were investigated with reference to the presence or absence of oxygen in the suspension. The breakdown products of the D1 protein were identified by immunoblotting with anti-D1 polyclonal antibodies which were proven to recognize mainly the C-terminal region of the protein. The results obtained show that (i) the light-induced inactivation of the photosystem II electron transport activity under anaerobic conditions is faster than in the presence of oxygen; (ii) depletion of D1 protein is observed on a longer time scale with respect to loss of electron transport activity and is faster when photoinhibition is performed in the presence of oxygen; (iii) C-terminal fragments of D1 are only observed when photoinhibition is carried out anaerobically and are mainly localized in the stroma-exposed regions; and (iv) the fragments observed after anaerobic photoinhibition are quickly degraded on further illumination of the thylakoid suspension in the presence of oxygen.

Light-induced inhibition of oxygenic photosynthesis is a widely documented phenomenon (Powles, 1984). At the molecular level, the target of photoinhibition is photosystem II (PSII),[1] whose electron transport activity is severely impaired by high-intensity illumination. In the PSII reaction center all the redox components necessary for the primary photochemical events are associated with the heterodimer formed by the two proteins D1 and D2 (Nanba & Satoh, 1987; Barber et al., 1987). The D1 protein plays a special role in the metabolic machinery of PSII, being characterized by a turnover higher than that of any other thylakoid polypeptide (Mattoo et al., 1981). Moreover, its degradation rate increases

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[1] Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPIP, 2,6-dichlorophenolindophenol; LHCP, the main light harvesting complex serving photosystem II; PAGE, polyacrylamide gel electrophoresis; NBT, nitro blue tetrazolium; pheo, pheophytin; PS, photosystem; P_{680}, primary electron donor in PSII; Q_A, first quinone acceptor in PSII; Q_B, second quinone acceptor in PSII; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tyr_Z, tyrosine 161 on D1 protein.

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during high light illumination (Kyle, 1985), and therefore, it has been proposed that photoinhibition of PSII results from a depletion of this polypeptide from thylakoid membrane (Arntzen et al., 1984). The light-induced degradation of D1 is thought to be triggered by one or more of the molecular events which bring about the structural and functional changes of the reaction center complex responsible for the impairment of the electron transport activity under photoinhibitory conditions. However, the mechanisms underlying PSII inactivation and D1 degradation have not been fully clarified [see Andersson and Styring (1991) for a recent review]. Current hypotheses include effects both on the acceptor (Kyle et al., 1984; Styring et al.; 1990; Setlik et al., 1990) and on the donor side (Theg et al., 1986). On the basis of the observation that the block by DCMU of the electron transport from the tightly bound plastoquinone Q_A to the second plastoquinone acceptor Q_B is partially preventing D1 degradation in the light, the Q_B site has been proposed as the primary site of photoinhibition (Kyle et al., 1984). Generation at this site of oxygen radicals, able to damage the protein, has been indicated as the primary cause for the light-induced breakdown of D1 protein (Kyle et al., 1984; Kyle, 1985). Involvement of reactive oxygen species in D1 degradation has been supported in recent experiments in which the protein was degraded in the dark in the presence of an artificial system able to generate hydroxyl radicals (Richter et al., 1990).

An alternative view on the participation of the acceptor side in photoinhibition suggests that, under strong illumination, the double reduction of Q_A (Styring et al., 1990) or the production of a stable form of Q_A^- (Setlik, et al., 1990) is the main perturbative event. The consequent increased charge recombination of the P_{680}^+–pheo$^-$ radical pair to the P_{680} triplet state is the source of singlet oxygen known to destroy P_{680} (Telfer et al., 1990).

As to the donor side, it is becoming clear that its inactivation makes the reaction center more susceptible to photoinhibition (Theg et al., 1986). Under these conditions the degradation of D1 protein is faster (Jegerschold et al., 1990) and is independent of the presence of oxygen, taking place also in its absence (Jegerschold & Styring, 1991). The presence of long-lived, highly oxidizing radical species such as Tyr_Z^+ or P_{680}^+ is thought to be the source of the irreversible changes ending with complete inactivation and breakdown of the reaction center D1 protein (Jegerschold et al., 1990; Shipton & Barber, 1991). Thus, the evidences so far available suggest that the photochemical processes leading to degradation of D1 may follow different pathways and oxygen may or may not be involved, depending on the particular conditions.

Whatever the primary damage induced by excessive light may be, the subsequent event is the depletion from the thylakoid membrane of the D1 protein (Arntzen et al., 1984; Ohad et al., 1985). Apart from the involvement of toxic radical species, the suggestion that degradation of D1 would be mediated by specific membrane-bound protease(s), possibly the same enzyme(s) involved in the physiological light-induced D1 turnover, has been proposed for some time (Ohad et al., 1985, 1990). This hypothesis has been strongly supported by the recent finding that light-induced D1 degradation does not occur in isolated thylakoids when photoinhibition is performed at low temperature, but degradation starts, even in the dark, as soon as the temperature is raised to 20 °C (Aro et al., 1990). Moreover, since the isolated reaction center is able to perform D1 degradation producing a fragmentation pattern similar to that observed in more complex systems (Shipton & Barber, 1991), the possible candidates for the proteolytic activity are restricted to the protein components of the reaction center itself. D1 fragments generated both during normal light-induced turnover and under photoinhibitory conditions have been only partially identified. In vivo, a single membrane-bound fragment of 23.5 kDa has been detected, thought to correspond to the N-terminal region of the protein (Greenberg et al., 1987), while other D1 fragments, generated during photoinhibition of isolated thylakoids (Aro et al., 1990), of detergent-derived oxygen-evolving PSII preparations (Virgin et al. 1990) and of the isolated PSII reaction center (Shipton & Barber, 1991), have been recently observed but not further characterized.

In this paper we report on the breakdown of D1 after photoinhibition with particular reference to the effect of the presence or absence of oxygen. For the first time we describe D1 fragments containing the C-terminal region of the protein, and their localization in the stroma lamellae is proven.

Materials and Methods

Thylakoid Isolation and Photoinhibition. Pea (*Pisum sativum* L., var. Progress n.9) were grown in a growth chamber at 24 °C, 400 $\mu E \cdot m^{-2} \cdot s^{-1}$, and 80% relative humidity. Pea thylakoids were isolated according to Aro et al. (1990) and resuspended at 200 μg of chl/mL in 50 mM Tricine, pH 7.8/0.1 M Sorbitol/5 mM $MgCl_2$/10 mM NaCl (incubation buffer). This suspension was utilized without further treatment to carry out aerobic photoinhibition. To obtain anaerobic conditions, thylakoids were resuspended in the incubation buffer, which had been extensively degassed. The suspension was flushed in the dark with nitrogen for 15 min. Just before light treatment 5 mM glucose, 100 μg/mL glucose oxidase, and 10 μg/mL catalase were added (Crystall et al., 1989). Light treatment was carried out at 20 °C in a jacketed, 2-mm-thick flat cuvette, using a light intensity of 6500 $\mu E \cdot m^{-2} \cdot s^{-1}$.

Membrane Fractionation. Grana and stroma membranes, both from aerobically and anaerobically photoinhibited thylakoids, were obtained by the digitonin-based method (Kyle et al., 1987), following the procedure described by Aro et al. (1990). After light treatment, digitonin was added to a final concentration of 0.2%, and after 2 min, solubilization was stopped by adding 10 volumes of incubation buffer. Grana and stroma thylakoids were isolated by differential centrifugation. In the case of fractionation of anaerobically photoinhibited thylakoids, all buffers were extensively degassed and flushed with nitrogen for 30 min before their use.

Isolation and Proteolysis of D1 from Wheat. D1 protein was isolated from PSII reaction center complex obtained as described by Dekker et al. (1989). Reaction center polypeptides were resolved by preparative SDS–PAGE/6 M urea (see below), and after a short staining of the gel in 0.5% Coomassie in 50% methanol, bands were cut out of the gel and electroeluted. Proteolysis of D1 protein with Lys-C specific protease was carried out at a protein concentration of about 0.5 mg/mL with 1.4 units/mL protease for 4 h at 37 °C.

SDS–PAGE and Immunoblot. SDS–PAGE in the presence of 6 M urea was performed according to Gounaris et al. (1988) using a 12–18% linear acrylamide gradient. For immunoblot experiments, the resolved proteins were transferred to a nitrocellulose filter (Millipore, 0.45 μm) according to Towbin et al. (1979). After suitable incubation with the primary antibodies, the immunocomplexes were detected using biotinilated goat anti-rabbit secondary antibody and extravidine-labeled alkaline phosphatase. NBT and BCIP were used as chromogenic substrates for alkaline phosphatase. The preparation of anti-D1 polyclonal used here has been previously

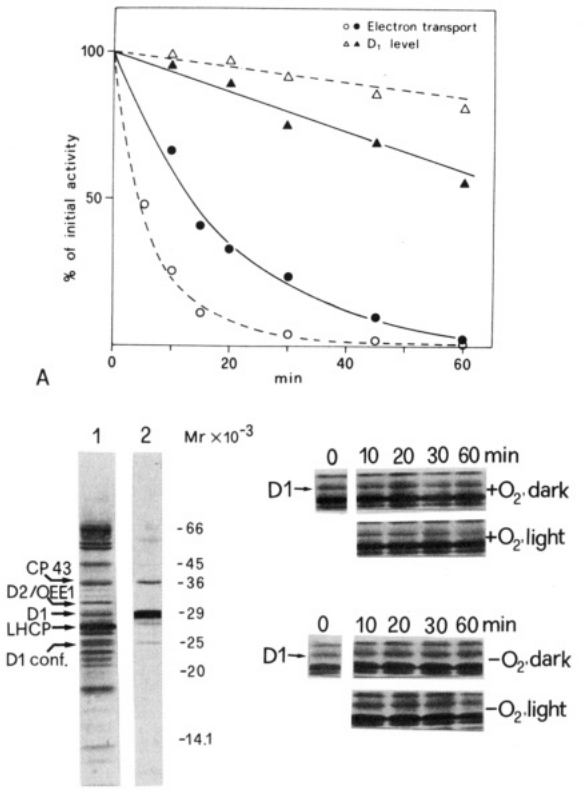

FIGURE 1: Photoinhibition of pea thylakoids. (A) Kinetics of electron transport impairment (circles) and loss of D1 protein (triangles) in the presence (closed symbols) or absence of oxygen (open symbols); (B) SDS–PAGE pattern of the polypeptide content of thylakoid membrane (lane 1) and identification of the D1 protein by immunoblot using anti-D1 antiserum (lane 2); (C) Coomassie-stained bands of the 25–35-kDa region of the SDS–PAGE after different times of illumination.

described (Di Paolo et al., 1990). A polyclonal against wheat CP43 was raised in rabbit using a pure CP43 fraction obtained as a byproduct of the wheat reaction center preparation. Immunization schedule and bleeding of rabbits were according to standard procedure.

Other Methods. Densitometric analysis of Coomassie-stained gels and immunodecorated blots were performed using a Shimadzu CS 930 densitometer. Chlorophyll concentration was estimated according to Arnon (1949). Electron transport activity from water to DPIP was measured as previously described (Giardi et al., 1990).

Results

Functional and Chemical Effects of Photoinhibition. The effect of exposing isolated pea thylakoids to an excess of white light on electron transport activity ($H_2O \rightarrow$ DPIP) and D1 protein level are shown in Figure 1A both in the presence and in the absence of oxygen. Approximately 13 min is required to cause 50% loss of electron transport activity in the presence of oxygen while in its absence 4 min is sufficient to achieve the same degree of inactivation. Identification of D1 polypeptide in our gel system has been carried out by immunoblot using a polyclonal anti-D1 antiserum. Comparison of lane 1 (SDS–PAGE) and lane 2 (immunoblot) of Figure 1B shows how D1 polypeptide in our gel system migrates as a band well resolved both from D2 (which is partially superimposed on the extrinsic 33-kDa polypeptide) and from the major antenna component LHCP. The presence of other polypeptides such as cyt*f* in the same region of the gel cannot be excluded. However, since their amount is independent of the exposure to light, a reliable densitometric quantification of the decreasing of D1 with illumination was possible. Figure 1C shows the profiles of polypeptides in the 25–35-kDa range for thylakoids which had been photoinhibited for different periods of time either in the presence or in the absence of oxygen. Using some of the polypeptides whose concentration is independent of the illumination time as internal standard (i.e., LHCP and α- and β-subunits of the coupling factor), we found that D1 depletion is more pronounced when photoinhibition is carried out in the presence of oxygen (Figure 1A). In fact, under aerobic conditions, the D1 level after a 60-min illumination was 55% of the dark control compared to 82% in anaerobically treated thylakoids subjected to the same illumination time. No loss of D1 protein could be observed in dark controls, whether oxygen was present or not.

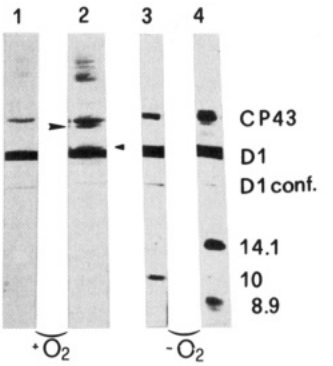

FIGURE 2: Immunoblot with anti-D1 polyclonal antiserum of dark control (lanes 1 and 3) and photoinhibited (lanes 2 and 4) thylakoids in the presence or absence of oxygen. The upper and lower arrows marked on lane 2 indicate respectively the supposed truncated D1/D2 heterodimer and the slower migrating form of D1 (see the Results and Discussion sections).

Detection of D1 Fragments. To investigate the fate of D1 during photoinhibition and to search for its proteolytic fragments, we performed immunoblot analysis using a polyclonal anti-D1 antiserum. The reactivity of our antibody toward whole thylakoid polypeptides is shown in Figure 2, lane 1: besides D1, the antibody recognizes a polypeptide having the same mobility as CP43 apoprotein, a 25-kDa band, possibly the D1 conformer (Greenberg et al., 1987), and a 55-kDa band, possibly representing the D1/D2 heterodimer (Marder et al., 1987). The nature of the cross-reaction of the antibody with CP43 is not clear at present since a contamination of our antigen with CP43 can be excluded (see below).

After a 30-min illumination in the presence of oxygen, two new bands are observed (Figure 2, lane 2). A first band is detected just below the CP43 apoprotein with an apparent molecular mass of 36–37 kDa. The possibility that this band could be related to CP43 was excluded by immunoblot using a polyclonal raised against CP43. Instead, a positive although weak reaction of this band with anti-D2 antiserum is detected (not shown). The second band looks like a small amount of D1 protein with slightly reduced electrophoretic mobility. Both of these bands, which have also been observed by others (Virgin et al., 1990; Callahan et al., 1990; Shipton & Barber, 1991), are present in a low amount, below the sensitivity of Coomassie staining. No lower molecular weight polypeptides deriving from D1 degradation were detected by our antibody, indicating their loss from the membrane or their further and rapid breakdown (Ohad et al., 1985; Wettern & Galling, 1985).

When photoinhibition was carried out in the absence of oxygen, a different pattern was observed after the immunoblot (Figure 2, lanes 3 and 4). Although in a lesser amount, the band at 36–37 kDa was still detected, while the small amount of D1 with reduced electrophoretic mobility was only occa-

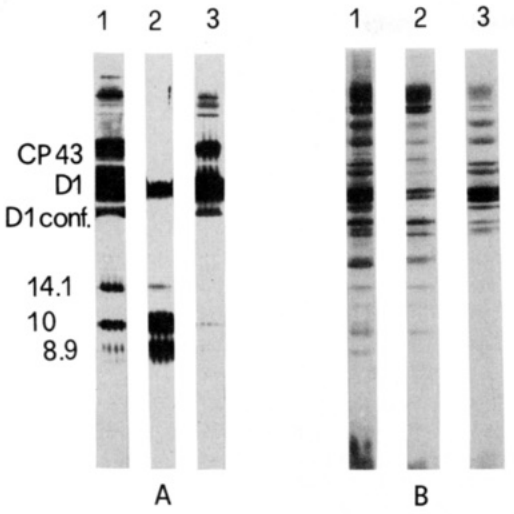

FIGURE 3: Immunoblot (A) and Coomassie-stained SDS gel (B) of stroma lamellae (lane 2) and grana membranes (lane 3) of anaerobically photoinhibited pea thylakoids. Unfractionated thylakoids are shown in lane 1 for comparison.

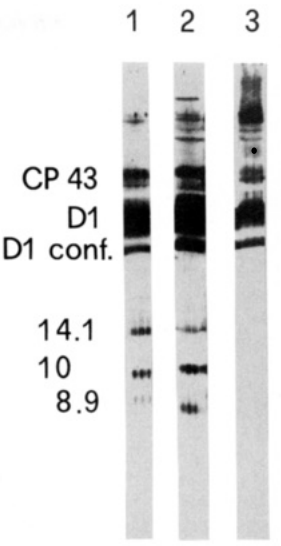

FIGURE 4: Immunoblot of anaerobically photoinhibited pea thylakoids (lane 1) and the same after a 30-min exposure to atmospheric oxygen in the dark (lane 2) or under illumination (lane 3).

sionally resolved. However, a major change in the immunoblot pattern is the appearance of some lower molecular weight bands: two new polypeptides are clearly detected at 14.1 and 8.9 kDa. An immunoreactive band at 10 kDa is also produced in the dark control whose origin was not further investigated (Figure 2, lane 3). Once again, the possibility that these fragments were related to CP43 was excluded by the lack of reaction with anti-CP43 polyclonal. We concluded that, at variance with the situation observed with aerobically induced photoinhibition, at least two D1 fragments are found in the membrane when oxygen is excluded from the thylakoid suspension subjected to photoinhibition.

Localization of the Fragments. Since localization of these fragments in the two compartments of the thylakoid membrane (i.e., grana partitions and stroma lamellae) is relevant to the elucidation of the mechanism of D1 degradation and turnover, we proceeded to the fractionation of anaerobically photoinhibited thylakoids into stroma lamellae and grana partitions using the digitonin-based method (Kyle et al., 1987). Figure 3A shows that virtually all of the D1 fragments observed in thylakoids (lane 1) are localized in the stroma membranes (lane 2), while only trace amounts are detected in the grana fraction (lane 3). The corresponding Coomassie-stained SDS–PAGE is shown for comparison in Figure 3B.

Effect of Oxygen. The results reported above seem to imply an important role for oxygen in determining the fate of D1 after photoinhibition. However, a recent investigation by Sundby (1990) suggests the possibility that the faster inactivation of PSII electron transport activity observed under anaerobic conditions is only apparent, the increased vulnerability being rather due to the removal of protecting bicarbonate from the medium. With the aim to check whether this could be the case also for degradation of D1 fragments, we exposed to air samples of anaerobically photoinhibited thylakoids (containing detectable D1 fragments) both in the dark (at 20 °C) and in the light (at 20 °C and 6500 $\mu E \cdot m^{-2} \cdot s^{-1}$). The results are presented in Figure 4 and show that when thylakoids that had been treated for 30 min in the light and in the absence of oxygen (lane 1) were subsequently equilibrated with air in the dark (lane 2), no significant changes were observed in the immunoblot. On the contrary, when exposure to air was performed in the light (lane 3), no trace of the low molecular weight D1 fragments could be observed, indicating their complete degradation. This experiment indicates that restoration of the atmospheric concentration of oxygen and carbon dioxide have no effect per se on the degradation of the fragments and that the action of light is required to ensure complete breakdown. An analogous experiment was performed by adding separately pure oxygen or a 20 mM solution of bicarbonate in deoxygenated buffer. Degradation of the fragments was only observed when oxygen was added and the thylakoid suspension was exposed to light.

Characterization of the Fragments. So far, the only characterized D1 fragment observed during light-induced D1 turnover is a 23.5-kDa polypeptide thought to correspond to the N-terminal region of the protein (Greenberg et al., 1987). Although the presence of other fragments has been recently reported (Aro et al., 1990; Virgin et al., 1990; Shipton & Barber, 1991), no correlation to a distinct part of the protein has been attempted. In our experiments we never detect fragments with a molecular mass higher than 14.1 kDa. This indicates that the immunoreactive bands in our blots must be either the result of a subsequent breakdown of the 23.5-kDa N-terminal fragment or different fragments representing possibly the C-terminal region of the protein. To clarify this point, we decided to better characterize the immunological properties of our anti-D1 antiserum, taking advantage of the known sequence of D1 protein from wheat. While D1 from pea (Oishi et al., 1984) and spinach (Zurawski et al., 1982) does not contain any lysine residue in its amino acidic chain, D1 from wheat contains a single lysine residue at position 238 (Marder et al., 1988). The latter protein can therefore be subjected to a highly specific proteolysis by Lys-C protease, which will produce two well-defined fragments, one corresponding to the N-terminal region of D1 and consisting of amino acids 1–238, the other corresponding to the C-terminal region and containing residues 239–353. We therefore isolated the reaction center complex from wheat using the method described by Dekker et al. (1989), and its polypeptides were resolved by SDS–PAGE (Figure 5, lane 1). After Coomassie staining, D1 was cut out of the gel and electroeluted. Its purity was assessed by SDS–PAGE (lane 2) and then treated with Lys-C protease. The D1 fragments were resolved again by SDS–PAGE (lane 3) and blotted onto nitrocellulose paper. The reactivity of our antibody toward the two fragments is shown in lane 4. Densitometric analysis of the stained gel and immunoblot indicate a much stronger (approximately 20-fold)

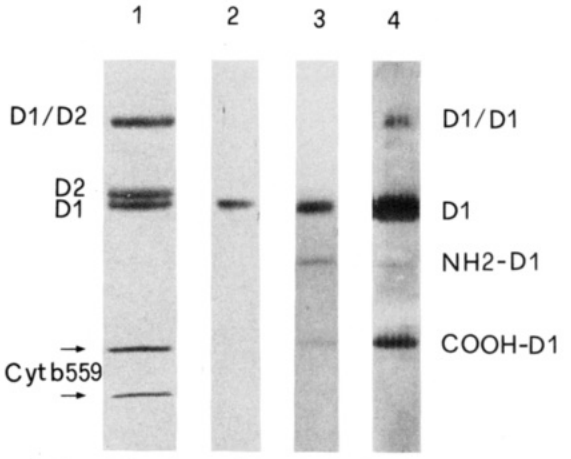

FIGURE 5: Reactivity of an anti-D1 polyclonal antiserum anti-D1 tested against the Lys-C fragments of D1 protein from wheat. Lanes 1–3 represent a Coomassie-stained urea/SDS–PAGE gel of PSII reaction center (lane 1), D1 as obtained by electroelution of D1 band (lane 2), and the same D1 band after partial proteolysis by Lys-C protease (lane 3). Lane 4 represents the immunoblot of the gel in lane 3 showing the reactivity of our polyclonal against the two D1 fragments (see text for details).

reaction toward the C-terminal fragment than toward the N-terminal one.

These findings indicate that all of the D1 fragments observed in our experiments are related to the C-terminal region of D1 since the sensitivity of the antibody toward the N-terminal region was lower than Coomassie staining. To check that Lys-C proteolysis had not destroyed any epitope on D1, we also digested D1 from wheat with different amounts of V8 protease. This proteolysis is thought to produce a higher molecular weight D1 fragment, by cleavage of the protein at a residue shifted toward the C-terminus with respect to the lysine 238 (Shipton et al., 1990, and references cited therein). This approach has been successfully utilized by Shipton et al. (1990) to map the main epitope recognized by their anti-D1 polyclonal on the hydrophilic loop connecting the fourth and the fifth helices of D1. In our case, at every concentration of protease utilized, we only detected low molecular weight fragments, indicating that no main epitopes for our polyclonal were present on the N-terminal region of the protein.

Discussion

Impairment of PSII activity is faster when photoinhibition is carried out under anaerobic conditions. Although this phenomenon has been attributed to the removal of the protective effect of bicarbonate during deoxygenation by nitrogen or argon flushing (Sundby, 1990), recent evidences suggest that the faster loss of PSII activity during anaerobic illumination could represent a defensive mechanism against irreversible damages induced by light (Kirilovsky & Etienne, 1991). In fact, it has been shown that anaerobic photoinhibition of isolated thylakoids is, at least to some extent, a reversible process, recovery being possible through a mechanism different from resynthesis of D1 protein (Hundal et al., 1990a). Moreover, photoinhibition in the absence of oxygen has been shown to induce an increase in F_0 (the fluorescence yield after dark adaptation) and inactivation of oxygen evolution, but no irreversible changes could be detected at the Q_B site as revealed by thermoluminescence measurements (Kirilovsky & Etienne, 1991). Accordingly, we observe more D1 degradation in thylakoids illuminated in the presence of oxygen, suggesting that during anaerobic illumination only a fraction of the inactivated PSII centers is further subjected to degradation, the other fraction being subjected to reversible inactivation without D1 damage.

A main factor of uncertainty when D1 degradation is studied using immunoblot technique with polyclonal antisera raised against the whole protein is the general lack of knowledge on the specificity toward different regions of the protein.

After the test of our anti-D1 antiserum against the Lys-C fragments of D1 from wheat we may assert that only the C-terminal fragments of D1 are recognized under the experimental conditions used. This finding is not surprising if the D1 conformation is considered as it emerges from hydropathy and helix-forming potential calculations (Kyle, 1985; Mattoo et al., 1989; Barber, 1989) and from studies with right-side-out and inside-out vescicles using site-directed antibodies (Sayre et al., 1986). In this picture, in fact, the two largest hydrophilic, and therefore more immunogenic, regions of the protein are located toward the end of the molecule forming a loop between the fourth and fifth helices (44 residues) and the C-terminal tail (68 residues).

After immunoblot of thylakoids which had been photoinhibited in the presence or in the absence of oxygen, the first change we could observe was the appearance of a cross-reacting band having a molecular mass of about 36–37 kDa. The nature of this band, whose presence has been reported also by Virgin et al. (1990) and Shipton and Barber (1991), is not completely understood. On the basis of its cross-reactivity with anti-D2 antiserum, it has been proposed to represent a heterodimer truncated in D1 (Virgin et al., 1990). However, in view of its low level of cross-reactivity with our anti-D2 antiserum and its low molecular weight, it is possible that both proteins (D1 and D2) are actually truncated.

A second effect observed with photoinhibited thylakoids was the appearance of a new anti-D1 immunoreactive band just above the D1 protein. Its appearance seems to depend on the presence of oxygen during illumination, but it was been occasionally resolved also when illumination was carried out anaerobically. It is not clear if this band has any relation with the D1* described by Callahan et al. (1990) or with the irreversibly modified form described by Ohad et al. (1990).

As a side issue, we may notice that the cross-reaction of our anti-D1 antiserum with CP43 apoprotein (or with a comigrating polypeptide) cannot be due to contamination of the antigen since the same cross-reaction has been observed in preliminary experiments using polyclonal antisera raised against a C-terminal D1 fragment obtained by Lys-C digestion of the wheat protein. Whether this observation can be interpreted as the existence of at least partially similar epitope(s) is under investigation.

Two main C-terminal fragments are observed after anaerobic photoinhibition. The smaller fragment shows an apparent molecular mass of 8.9 kDa. If compared with the C-terminal fragment generated by Lys-C proteolysis of wheat D1 protein, this fragment seems to have a dimension compatible with a proteolytic cleavage at the QEEE sequence as suggested by Shipton et al. (1990). The second C-terminal fragment observed in this work has an apparent molecular mass of 14.1 kDa, and therefore, it has to be produced by cleavage at a different site with respect to the previous one. We cannot say whether the two fragments are the result of two independent or of two subsequent cleavages.

An interesting result of this study is the total disappearance of the fragments when photoinhibition is performed in the presence of oxygen. Since photoinhibition under these conditions results in a significant loss of D1, we must conclude that the C-terminal fragments of D1 are either quickly de-

graded or released from the membrane. The second possibility seems to be ruled out by the fact that no immunoreactive bands were detected in the supernatant after precipitation with 5% TCA or 80% acetone. Moreover, since addition of oxygen and/or bicarbonate in the dark to a sample of thylakoids previously photoinhibited under anaerobiosis does not induce further breakdown of the fragments, we may conclude that the effects of light and oxygen are not independent but they must be present at the same time to induce complete disappearance of the fragments. The absence of D1 fragments after aerobic photoinhibition has recently been reported also for the isolated PSII reaction center (Shipton & Barber, 1991). Therefore, we conclude that the complete degradation observed in the presence of oxygen and illumination is due to activated oxygen species. Photoinhibition involving the acceptor side is thought to produce toxic oxygen radicals, which may be responsible for the complete degradation we observed. The same mechanism has been proposed for the further breakdown of the N-terminal fragment of D1 which was found to be stable in the dark but quickly degraded under illumination (Greenberg et al., 1987). Rather surprisingly the combined effect of oxygen and light seems to be specific for D1 fragments since other PSII polypeptides are not affected at least in the time scale considered. A possible explanation could be a higher accessibility of the fragments to the oxygen radicals with respect to the other PSII proteins which remain associated in the PSII complex where a more effective protection could be exerted by specific carotenoid molecules (Griffith et al., 1955). On the other hand, the presence of D1 fragments after illumination under anaerobic conditions strongly indicates that oxygen is not involved in primary breakdown of D1 protein. Very recently Jegerschold and Styring (1991) have reported the presence of a D1 fragment in anaerobically photoinhibited thylakoids when photoinhibition is performed after inactivation of the donor side. Under these conditions, abnormally long-lived highly oxidizing radicals P_{680}^+ and Tyr_Z^+ are thought to be induced at the donor side, which may be responsible for D1 damaging. The fragment detected by these authors is probably related to that described in the present work, corresponding to the C-terminal region of the protein. Although in our case the donor side has not been purposely inhibited, a partial inactivation of the water-splitting enzyme cannot be excluded in view of the pH at which the experiment was performed (pH 7.8).

The finding that the D1 fragments are mainly localized in the stroma-exposed membranes raises some questions about the changes in the structural organization of PSII during photoinhibition. It has been previously shown that photoinhibition brings about the migration from grana membranes to stroma-exposed lamellae of some of the PSII polypeptides (Hundal et al., 1990b). An increase of 100% of CP43 and CP47 polypeptides has been found in stroma membranes isolated from photoinhibited thylakoids, and a lesser increase has been reported for D2 and cytb_{559}. These data suggest that after D1 damaging, disassembly of PSII takes place in the grana membranes and then the disassembled polypeptides migrate to stroma lamellae. If this is the case, taking into account that D1 fragments are observed mainly in stroma-exposed membranes, there is a possibility that D1 protein is degraded in this membrane compartment. However, the recent finding that the isolated PSII reaction center is able to perform D1 degradtion giving rise to a main fragment of about 24 kDa (Shipton & Barber, 1991), possibly the same as that produced during normal light induced turnover, strongly suggests that the D1 breakdown takes place when the polypeptide is still assembled in the reaction center complex. Thus, an alternative possibility is that after cleavage the C-terminal fragments migrate quickly to stroma-exposed membranes, where they are further metabolized. Migration to stroma lamellae of other PSII subunits may be the first step in the repair cycle. In fact, newly synthesized D1 polypeptides are known to be inserted in the stroma lamelle (Mattoo & Edelman, 1987), where they possibly assemble with the other PSII subunits in new PSII complexes (Ohad et al., 1990; Adir et al., 1990).

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Role of C6 Chirality of Tetrahydropterin Cofactor in Catalysis and Regulation of Tyrosine and Phenylalanine Hydroxylases†

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ABSTRACT: The chiral specificities of bovine striatal tyrosine hydroxylase (TH) (unphosphorylated and phosphorylated by cAMP-dependent protein kinase) and rat liver phenylalanine hydroxylase (PH) were examined at physiological pH using the pure C6 stereoisomers of 6-methyl- and 6-propyl-5,6,7,8-tetrahydropterin (6-methyl-PH_4 and 6-propyl-PH_4) and (6*R*)- and (6*S*)-tetrahydrobiopterin (BH_4). Both PH and phosphorylated TH have substantially higher V_{max} values with the unnatural (6*R*)-propyl-PH_4 than the natural (6*S*)-propyl-PH_4 (approximately 6- and 11-fold, respectively). However, the K_m's are also higher such that V_{max}/K_m is almost unaffected by C6 chirality. Unphosphorylated TH has equal K_m values for both isomers of 6-propyl-PH_4, but has about a 6 times greater V_{max} with the unnatural isomer, making it the fastest cofactor yet for this form of the enzyme. With the shorter 6-methyl group, chiral differences are still recognized by phosphorylated TH but hardly at all by PH. Inhibition of both PH and TH by amino acid substrate which occurs with (6*R*)-BH_4 as cofactor is also observed with (6*S*)-propyl-PH_4 but not with (6*S*)-BH_4, (6*R*)-propyl-PH_4, or (6*R*)- or (6*R*,*S*)-methyl-PH_4. The K_m for (6*S*)-BH_4 with phosphorylated TH is nearly 3 times higher than with (6*R*)-BH_4, but V_{max} is unchanged. With unphosphorylated TH, (6*S*)-BH_4 produces very low decelerating rates, which was shown not to be due to irreversible inactivation of the enzyme. The K_m for (6*R*)-BH_4 with either hydroxylase is 10 times higher than for the equivalently configured (6*S*)-propyl-PH_4. Comparison of these two cofactors reveals that the 1′ and 2′ side-chain hydroxyl groups of the natural cofactor promote different regulatory functions in PH than in TH.

Although almost all of the function necessary to support the chemical steps of the aromatic amino acid hydroxylases reside in the tetrahydropteridine ring of their common cofactor (6*R*)-tetrahydrobiopterin [(6*R*)-BH_4][1] (Figure 1), it is becoming increasingly clear that the (6*R*)-L-*erythro*-dihydroxypropyl side chain is critical for many aspects of enzyme regulation (Kaufman & Fisher, 1974; Kaufman & Kaufman, 1985; Shiman, 1985; Bailey et al., 1990b). These phenomena,

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[1] Abbreviations: BH_4, tetrahydrobiopterin (6-[L-erythro-1′,2′-dihydroxypropyl]-5,6,7,8-tetrahydropterin); (6*R*)-BH_4, 6*R* diastereoisomer of BH_4 (the natural isomer); (6*S*)-BH_4, 6*S* diastereoisomer of BH_4 (the unnatural isomer); PH_4, tetrahydropterin; DOPA, dihydroxyphenylalanine; HPLC, high-pressure liquid chromatography; Na_2EDTA, ethylenediaminetetraacetic acid disodium salt; DHPR, dihydropteridine reductase.

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