Acceptor Side Mechanism of Photoinduced Proteolysis of the D1 Protein in Photosystem II Reaction Centers†

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ABSTRACT: A 23-kDa breakdown product, containing the N terminus of the D1 protein, has been detected after photoinhibitory treatment of isolated photosystem II (PSII) reaction centers. The ability to induce charge separation in the reaction center and the presence of oxygen seem to be required for the generation of this fragment. It is suggested that, under these conditions, the initial light-induced damage to the complex occurs via singlet oxygen generated by the P680 triplet state and contrasts with the situation when an electron acceptor is present and donor-side photoinhibition gives rise to a 24-kDa C-terminal fragment of the D1 protein. The temperature sensitivity of the appearance of the 23-kDa N-terminal fragment suggests that the cleavage is not by a direct photochemical process but that it is proteolytic in nature, being triggered possibly by a conformational change induced by singlet oxygen-mediated photodestruction of the P680 chlorophylls. The existence of an intrinsic serine-type protease, within the reaction center itself, is supported by inhibition of the appearance of the 23-kDa N-terminal fragment by stoichiometric levels of soybean trypsin inhibitor. It seems likely that the 23-kDa N-terminal fragment which we have detected is the same as that identified *in vivo* by Greenberg et al. [Greenberg, B. M., Gaba, V., Mattoo, A. K., & Edelman, M. (1987) *EMBO J. 6*, 2865–2869] and originates from the acceptor-side mechanism advocated by Vass et al. [Vass, I., Styring, S., Hundal, T., Koivuniemi, A., Aro, E.-M., & Andersson, B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1408–1412].

It is well accepted, from analogies with purple photosynthetic bacteria (Barber, 1987; Michel & Deisenhofer, 1988) and from direct biochemical studies (Nanba & Satoh, 1987), that the photochemical reactions of photosystem II (PSII)[1] take place in a reaction center composed of the D1 and D2 proteins. Recognition of this has intensified the need to understand why, and by what means, the D1 protein rapidly turns over in illuminated intact systems (Mattoo et al., 1989). Current feeling is that the turnover of the D1 protein is due to a damage–repair cycle, inevitable because of the toxic nature of the photochemistry performed by PSII (Barber & Andersson, 1992). Furthermore, it is usually assumed that the process of photoinhibition becomes evident when the rate of damage is not matched by the rate of repair (Prasil et al., 1992).

Greenberg et al. (1987) were the first to show that *in vivo* the photoinduced turnover of the D1 protein gives rise to a 23.5-kDa breakdown fragment. On the basis of proteolytic mapping, they concluded that this fragment contained the N terminus of the D1 protein and, therefore, resulted from cleavage on the electron acceptor side of PSII, in the loop region linking putative membrane spans IV and V (Barber, 1987). Although they did not locate the precise cleavage site, they noted that this loop contained a region rich in glutamate, serine, and threonine residues which could be α-helix destabilizing. Indeed, such regions are characteristic for rapidly degrading proteins of eukaryotic organisms (Rogers et al., 1986).

Since this initial work, a number of related studies have been carried out employing various *in vitro* systems. Using isolated PSII core preparations, able to evolve oxygen, it has been shown that photoinhibitory illumination generates several D1 protein breakdown products, one being a 23-kDa N-terminal fragment (Virgin et al., 1990; Salter et al., 1992) assumed to be similar to that observed by Greenberg et al. (1987). This 23-kDa fragment was observed most clearly under conditions when water splitting and oxygen evolution occurred and, therefore, was attributed to damage induced by an overreduction on the acceptor side of PSII (Vass et al., 1992). If, however, water splitting activity was inhibited, then a different breakdown pattern of the D1 protein was observed. In particular, a fragment with an apparent molecular mass of 24 kDa was seen which contained the C-terminal portion of the D1 protein (De Las Rivas et al., 1992). A 24-kDa C-terminal D1 protein fragment has also been detected after photoinhibitory treatment of isolated PSII reaction centers of minimal composition (D1/D2/cyt *b559*/PsbI complex) illuminated in the presence of an added electron acceptor (Shipton & Barber, 1991; Barbato et al., 1991). These studies using the isolated PSII reaction center, however, did not seem to reveal the photoinduced production of a 23-kDa N-terminal fragment.

In this paper we show that, by using appropriate experimental conditions, we can generate a 23-kDa N-terminal primary fragment of the D1 protein by treating isolated reaction centers with photoinhibitory light. We attribute the generation of this protein fragment to the acceptor-side mechanism of photoinhibition (Barber & Andersson, 1992). As in the case of the generation of the 24-kDa C-terminal fragment (Shipton & Barber, 1991, 1992), the 23-kDa

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[1] Abbreviations: cyt *b559*, cytochrome *b*-559; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; Mes, 2-(*N*-morpholino)ethanesulfonic acid; $^1O_2$, singlet oxygen; P680, primary electron donor of PSII; Pheo, pheophytin; PSII, photosystem II; PsbI, product of the *psbI* gene; Q_A, first stable plastoquinone electron acceptor of PSII; SBTI, soybean trypsin inhibitor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SiMo, silicomolybdate.

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N-terminal fragment seems to be produced by a proteolytic activity contained within the reaction center itself. However, unlike the degradation reaction of the D1 protein to the 24-kDa fragment, the 23-kDa N-terminal fragment is only observed when oxygen is present.

MATERIALS AND METHODS

Isolation of PSII Reaction Centers and Photoinhibitory Illumination. The PSII reaction center, consisting of the D1 and D2 proteins, the α- and β-subunits of cytochrome *b*-559 and the product of the *psb*I gene, was isolated from pea thylakoid membranes as detailed previously (Chapman et al., 1991). The chlorophyll concentration was calculated by the method of Arnon (1949). The samples were suspended, depending on the experiment, either in 50 mM Mes–NaOH (pH 6.0) or in 50 mM Tris-HCl (pH 8.0). In both cases, 2 mM dodecyl maltoside and 0.2 M sucrose were added to prevent protein aggregation and to maintain maximum stability of the samples during the experimental periods.

Illumination of PSII reaction centers in solution was carried out with heat-filtered white light in a temperature-controlled cuvette holder with continuous slow stirring. Light intensities, chlorophyll concentrations, and other conditions are described in the figure legends.

Polypeptide Separation and Immunodetection. Solubilization of the reaction center samples was performed immediately upon completion of the treatment, and the polypeptide composition was analyzed by gradient SDS–PAGE (10–17% polyacrylamide) containing 6 M urea. The separated protein profiles were transferred onto nitrocellulose (Marder et al., 1987; Towbin et al., 1979) and immunodecorated with D1 antisera. Four different anti-D1 polyclonal antisera were utilized: **anti-D1**, raised against the product of the *psb*A gene (Nixon et al., 1987); **anti-D1c** (C-terminal specific), raised to a synthetic oligopeptide of the D1 protein molecule corresponding to residues 333 to the carboxy terminus (kind gift of Dr. P. J. Nixon and Dr. B. A. Diner); **anti-D1n1**, N-terminal specific antiserum raised to the N-terminal portion of the D1 protein molecule, generated by lysine specific endoproteolysis of the wheat D1 protein (see Figure 9a) (Barbato et al., 1991) (kind gift of Dr. R. Barbato); **anti-D1n2**, N-terminal specific antiserum, raised against a synthetic oligopeptide of the D1 protein corresponding to residues 32–87 (kind gift of Dr. J. A. Mullet). Alkaline phosphatase-conjugated secondary antibodies (Sigma) were then employed, followed directly by the appropriate chromogenic substrates. Western blots were quantified by 2D-scanning using a Hirschmann Elscript 400 densitometer.

Absorption Changes and Electron Transport Measurements. Absorption spectra of the samples were measured in a dual-beam spectrophotometer (Aminco, SLM Instrument DW2000) set with a bandwidth of 2 nm. The samples were maintained at 10 °C during the spectroscopic measurements. Electron transport rates of the reaction centers were measured as previously described (Chapman et al., 1988), using 0.5 mM $MnCl_2$ as an electron donor and 0.25 mM silicomolybdate (SiMo) as an electron acceptor, in 50 mM Tris-HCl buffer (pH 8.0) plus 2 mM dodecyl maltoside.

RESULTS

Generation of a 23-kDa N-Terminal D1 Protein Fragment in Isolated Photosystem II Reaction Centers. It has been shown previously that when isolated PSII reaction centers are exposed to illumination in the presence of artificial electron acceptors, degradation of the D1 protein gives rise to a 24-kDa fragment containing the C-terminal region of the D1 molecule (Shipton & Barber, 1991; Barbato et al., 1991). Under these conditions, secondary oxidation processes can occur within the complex, due to the lengthening of the $P680^+$ lifetime, resulting in the irreversible bleaching of pigments (Telfer et al., 1990, 1991). If, however, no artificial electron acceptors are present, then the $P680^+$ lifetime is shorter due to rapid back-reactions with reduced pheophytin ($Pheo^-$). This charge recombination occurs partly via the singlet excited state of P680, leading to an emission of chlorophyll fluorescence (Crystall et al., 1989), and partly via the P680 triplet state (Takahashi et al., 1987; Durrant et al., 1990). From flash absorption studies, it was clearly shown that the P680 triplet state lifetime was significantly shortened in the presence of oxygen (Durrant et al., 1990). This quenching is believed to result in singlet oxygen formation and to be responsible for the selective degradation of the P680 chlorophylls (Telfer et al., 1991). As a consequence of this, there is a corresponding loss of radical pair formation (Crystall et al., 1989; Durrant et al., 1990) and electron transport activity (Chapman et al., 1989). If oxygen is excluded, no such pigment degradation is observed (Telfer et al., 1990) and the isolated complex maintains its photochemical activity (Durrant et al., 1990; Chapman et al., 1989; McTavish et al., 1989).

Previously, no breakdown fragments of the D1 protein had been observed when isolated reaction centers were illuminated aerobically, in the absence of added electron acceptors, even though the level of D1 protein decreased (Shipton & Barber, 1991; Chapman et al., 1989). However, on addition of an electron donor (e.g., Mn^{2+}), we did observe the appearance of a breakdown fragment having an apparent molecular mass of 23 kDa (Ponticos et al., 1993). Figure 1a shows that a 23-kDa photoinduced fragment can also be observed when isolated reaction centers are illuminated in the absence of any electron donor or acceptor, when the assay is carried out at a lower pH (pH 6.0) and at a lower light intensity than that previously adopted. It is possible, therefore, that in our previous work (Shipton & Barber, 1991) further degradation of any D1 protein fragments produced at pH 8.0 in the absence of exogenous electron donors or acceptors took place extremely rapidly, thus preventing their detection. The immunoblot in Figure 1a shows that the anti-D1n1 antibody also detects the appearance of a 16-kDa fragment and the buildup of a 41-kDa band. Similar bands have been reported previously (Virgin et al., 1990; Shipton & Barber, 1991; Barbato et al., 1991; Salter et al., 1992). The 16-kDa fragment is likely to result from a secondary cleavage in a mid-position of the D1 protein (Barbato et al., 1991; Ponticos et al., 1993), while the 41-kDa band has been identified as an aggregate of the D1 protein and the α-subunit of cyt *b559* (Barbato et al., 1992a). The high molecular mass band at around 60 kDa is observed in untreated, as well as illuminated, samples and represents dimeric forms of the D1 and D2 proteins (Marder et al., 1987). Because of is diffuse nature, it is difficult to assess any quantitative change in the level of this band as a consequence of illumination.

Under experimental conditions identical to those used to generate the various immunodetectable bands of Figure 1a, there was a selective irreversible loss of chlorophylls that absorb maximally at 680 nm (see Figure 1b) and which are thought to constitute the primary electron donor, P680 (Telfer et al., 1990). Destruction of the P680 chlorophylls can be seen to occur very rapidly upon commencement of the illumination treatment. It is therefore possible that this bleaching of the primary electron donor is the triggering process which initiates

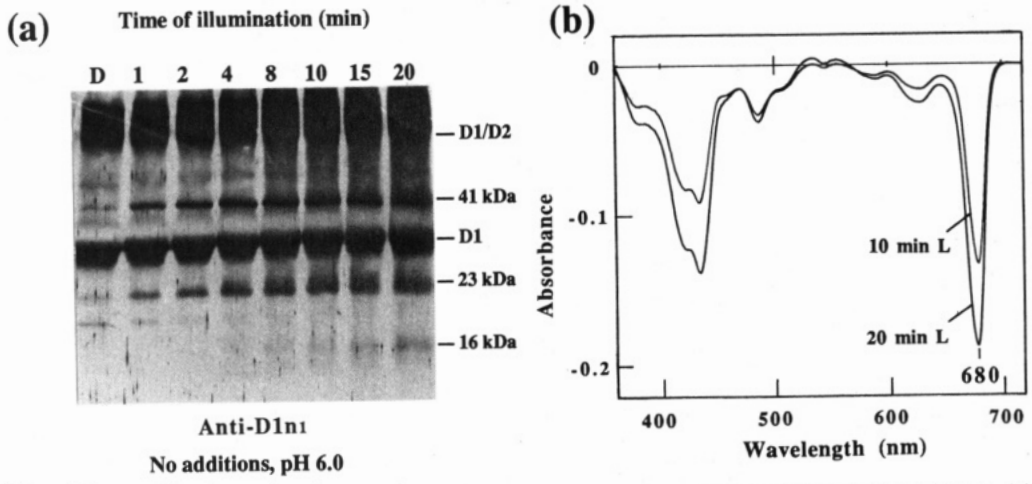

FIGURE 1: Protein and irreversible absorption changes detected in isolated PSII reaction centers after light treatment. (a) Western blot of the reaction centers showing a time course of illumination from 1 to 20 min. Samples were suspended at 50 μg mL^{-1} chlorophyll in 50 mM Mes–NaOH (pH 6.0) in the presence of 2 mM dodecyl maltoside, with no addition of electron donor or acceptor. Illumination was carried out with white light (2000 microeinstein m^{-2} s^{-1}), at 21–24 °C. D1 protein related bands were detected with an N-terminal specific D1 antibody (anti-D1n1). Lane D is dark control sample. (b) Irreversible difference absorption spectra (light-treated minus dark) corresponding to the samples shown in (a) illuminated for 10 and 20 min, respectively. The absorption change was measured in aliquots diluted 10-fold in Mes buffer (5 μg mL^{-1} chlorophyll).

cleavage of the D1 protein under these experimental conditions, although the precise interrelationship has yet to be established. It is worthy note, however, that in the experiment shown about 50% of the P680 chlorophyll absorbance has been lost after 20 min of illumination, while the level of the D1 protein does not seem to have decreased by an equal amount. This lack of correlation becomes more obvious as the temperature is lowered.

Figure 2 shows the result of a similar experiment to that presented in Figure 1a except that selective D1 polyclonal antisera were employed to enable the origin of the light-induced bands to be further assessed. It can be seen that the 23-kDa fragment is only observable with the N-terminal sensitive D1 antibody (anti-D1n2), while the 24-kDa fragment, generated by illuminating in the presence of 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) at pH 8.0, is only detected by the C-terminal sensitive antibody (anti-D1c). These results indicate that the 23-kDa photoinduced fragment is derived from a different part of the D1 protein than the 24-kDa fragment. The light intensities and pH used for production of the D1 protein fragments (N- and C-terminal) shown in Figure 2 were chosen to enable the level of fragments to be optimized. At lower light intensities and at pH 6.0, we have previously demonstrated that the 24-kDa C-terminal D1 fragment can be detected after illumination in the presence of 200 μM DBMIB without the appearance of the 23-kDa N-terminal band (Shipton & Barber, 1992). In accordance with the work of Barbato et al. (1992a), the 41-kDa band is detected by both antibodies, suggesting that it contains the entire D1 protein molecule.

Effect of Oxygen, Light Intensity, and Temperature on 23-kDa Fragment Production. The generation of the 24-kDa C-terminal fragment of the D1 protein has been shown to be independent of oxygen (Shipton & Barber, 1991, 1992), but as Figure 3 clearly shows, the appearance of the 23-kDa N-terminal fragment is only observed under aerobic conditions. Similarly, the photoinduced irreversible loss of the P680 chlorophylls and inhibition of photochemical activity observed with isolated PSII reaction centers with no addition have been shown to require the presence of oxygen (Telfer et al., 1990). Moreover, as Figure 3 shows, under anaerobic conditions formation of the adduct of the D1 protein and α-subunit of

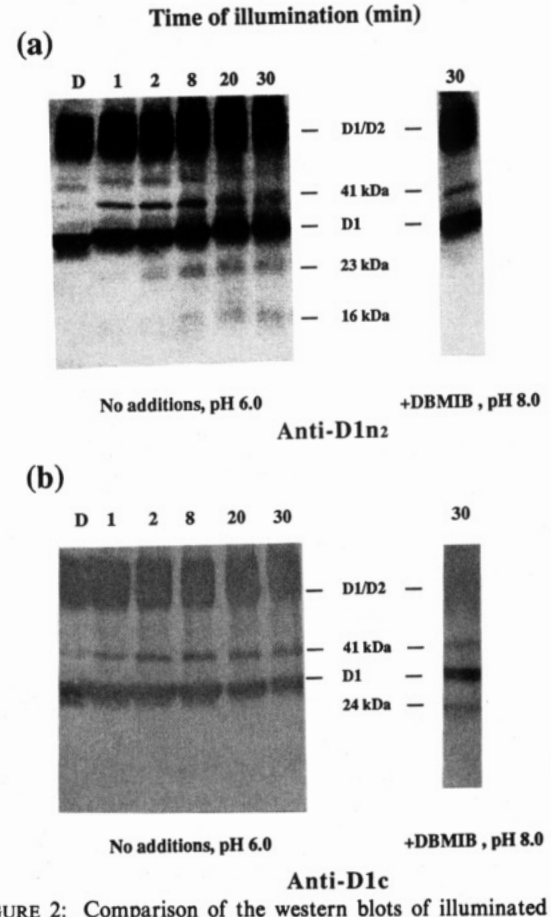

FIGURE 2: Comparison of the western blots of illuminated PSII reaction center samples detected with two different specific antibodies for the D1 protein: (a) N-terminal specific D1 antibody (anti-D1n2); (b) C-terminal specific D1 antibody (anti-D1c). The samples were illuminated with white light (2000 microeinstein m^{-2} s^{-1}) for 1, 2, 8, 20, and 30 min in 50 mM Mes–NaOH (pH 6.0) in the presence of 2 mM dodecyl maltoside, with no addition of electron donor or acceptor. On the right, aliquots are shown of samples illumianted with white light (6000 microeinstein m^{-2} s^{-1}) in 50 mM Tris-HCl (pH 8.0) in the presence of 2 mM dodecyl maltoside and 200 μM DBMIB as an electron acceptor. Chlorophyll concentration and temperature are as in Figure 1a.

cyt *b559* at 41 kDa is severely retarded, and the D1 protein does not show an upward shift in its apparent molecular mass

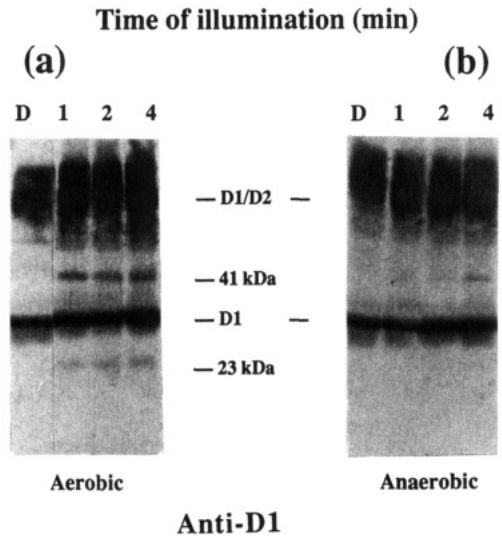

FIGURE 3: Western blots showing the light-induced production of the 23-kDa D1 protein degradation product in isolated PSII reaction centers in the presence or absence of oxygen. PSII reaction centers were suspended in 50 mM Mes–NaOH (pH 6.0) with 2 mM dodecyl maltoside (no electron donor or acceptor). Samples were illuminated for 1, 2, or 4 min. Chlorophyll concentration, light intensity, and temperature are as in Figure 1a. D1 protein bands were detected with a D1 antibody raised against the whole protein (anti-D1). Anaerobic conditions (b) were achieved by the addition of 10 mM glucose, 0.2 mg mL^{-1} glucose oxidase, and 0.2 mg mL^{-1} catalase; the cuvette was extensively flushed with nitrogen prior to illumination.

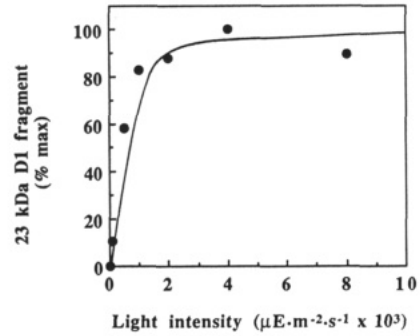

FIGURE 4: Effect of light intensity (100–8000 microeinstein m^{-2} s^{-1}) on the production of the 23-kDa D1 protein fragment. Other conditions for the treatment are as described in Figure 1a. D1 protein related bands were detected with a D1 antibody raised against the whole protein (anti-D1) and quantified by 2D densitometry.

typically seen as a result of photoinhibitory damage (e.g., see Figure 2a).

We have also found that the formation of the 23-kDa N-terminal fragment occurs as a result of relatively mild light treatments (see Figure 4), bearing in mind that the isolated reaction center contains a low level of antenna pigment molecules. This is again in contrast to the production of the C-terminal 24-kDa fragment, which requires a much higher dose of irradiation (Shipton & Barber, 1992). Furthermore, the appearance of the 23-kDa fragment was found to be severely restricted by cold treatment as indicated in Figure 5 and contrasts with the insensitivity of the photoinduced bleaching of the P680 chlorophylls to low temperature. At room temperature, the appearance of the 23-kDa N-terminal fragment (generated during illumination without exogenous electron donors or acceptors, pH 6.0) showed a similar time course to that of the photoinduced loss of electron transport activity (see Figure 6), assayed by monitoring the ability of the isolated PSII reaction center to reduce silicomolybdate

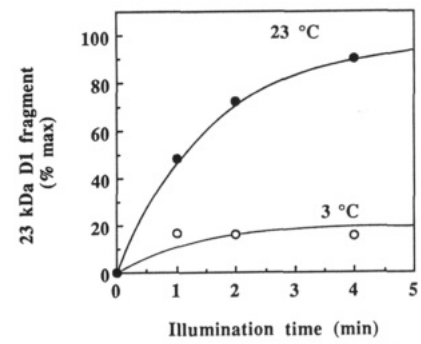

FIGURE 5: Graph showing the temperature dependency of the 23-kDa D1 protein fragment production. Illumination was performed at two different temperatures (2–4 and 21–24 °C). The other conditions for the treatment are as described in Figure 1a. D1 protein related bands were detected with a D1 antibody raised against the whole protein (anti-D1) and quantified by 2-D densitometry.

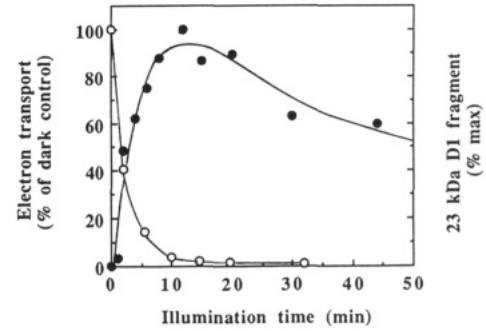

FIGURE 6: Comparison of the kinetics of photoinactivation of the PSII reaction center electron transport (open circles) with the kinetics of photoinduced production of the 23-kDa D1 protein fragment (solid circles). PSII reaction centers were suspended in buffer with no added electron donors or acceptors and illumination treatments performed as described in Figure 1a. At the required times two aliquots were taken, one for measurement of the electron transport rate and the other for analysis by western blotting. The measurement of the electron transport activity was performed after the dilution of the aliquot into 50 mM Tris-HCl (pH 8.0) containing 0.5 mM $MnCl_2$ and 0.25 mM SiMo. D1 protein related bands were detected with a D1 antibody raised against the whole protein (anti-D1). The electron transport activity was measured as the initial light-induced rate of silicomolybdate reduction using Mn^{2+} as an electron donor, after dilution to a chlorophyll concentration of 5 μg mL^{-1}. The 100% value of $MnCl_2$ to SiMo electron transport activity in reaction centers prior to illumination was 4800 μequiv electrons (mg of Chl)$^{-1}$ h^{-1}.

using Mn^{2+} as an electron donor. Although at room temperature this apparent relationship could be observed, at low temperature loss of photochemical activity occurred without the appearance of the 23-kDa fragment, indicating that the protein cleavage is not directly correlated to a photochemical event. These different responses to low temperature support the concept that degradation of the D1 protein is a secondary process triggered by inactivation of the photochemical reactions and is enzymatic in nature (Aro et al., 1990). A similar conclusion has been reached based partly on differential temperature effects and partly on the action of protease inhibitors, both for the 24-kDa C-terminal fragments generated in isolated reaction centers (Shipton & Barber, 1992) and for an N-terminal 23-kDa fragment produced in larger PSH complexes (Salter et al., 1992; Virgin et al., 1991).

Evidence for Proteolytic Activity within the Reaction Center Complex Involved in the Production of the 23-kDa Fragment. In Figure 7 we show that the appearance of the 23-kDa N-terminal fragment can be blocked by the addition of soybean trypsin inhibitor (SBTI), suggesting in the involvement of a serine-type protease in this process. Total inhibition of the formation of the 23-kDa fragment is observed when the

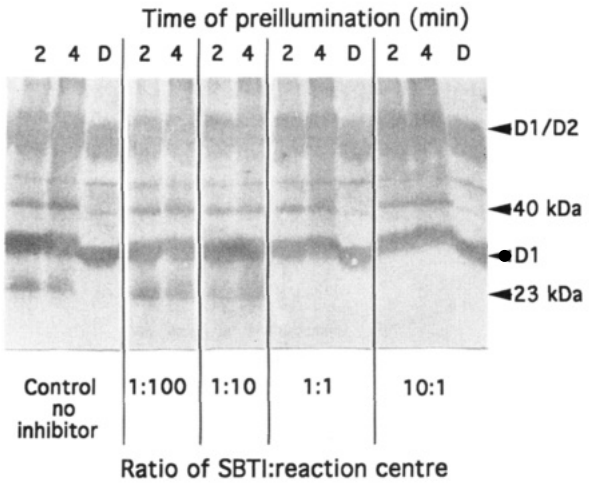

FIGURE 7: Detection and quantification of serine-type proteolytic activity associated with PSII reaction centers resulting in 23-kDa D1 protein fragment formation. Isolated reaction centers (RC) were diluted to 50 μg mL^{-1} chlorophyll in 50 mM Mes–NaOH (pH 6.0) in the presence of 2 mM dodecyl maltoside with a range of concentrations of soybean trypsin inhibitor (SBTI): 100 μM (ratio SBTI:RC 10:1), 10 μM (1:1), 1 μM (1:10), and 0.1 μM (1:100). Samples were incubated in the dark for 5 min, prior to illumination for 2 and 4 min at 21–24 °C with white light of 2000 microeinstein m^{-2} s^{-1} intensity. Aliquots were taken as required for immediate solubilization. The control sample contained no inhibitor. The lanes labeled D were incubated for 4 min in darkness before solubilization (in the presence of inhibitor as indicated). D1 protein related bands were detected with a D1 antibody raised against the whole protein (anti-D1).

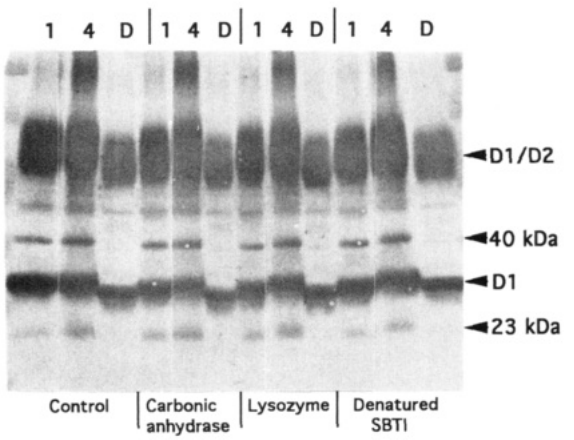

FIGURE 8: Western blots showing the level of light-induced 23-kDa D1 protein fragment in isolated PSII reaction centers in the presence of various added proteins. Samples were diluted to 50 μg mL^{-1} chlorophyll in 50 mM Mes–NaOH (pH 6.0) and 2 mM dodecyl maltoside. Either no additions were made (control) to these samples, or carbonic anhydrase, lysozyme, or denatured SBTI was added in an equimolar concentration to the reaction center complexes (10 μM). Samples were incubated in the dark for 5 min prior to illumination for 1 and 4 min at 21–24 °C with white light of 2000 microeinstein m^{-2} s^{-1} intensity. Aliquots were solubilized immediately after being removed from the illumination cuvette. Lanes labeled D wre incubated in the dark for 4 min in the presence of the added protein before solubilization. D1 protein related bands were detected with a D1 antibody raised against the whole protein (anti-D1).

protease inhibitor is added at stoichiometric levels with the reaction center, indicating that the proteolytic activity is unlikely to be due to any minor contaminants such as soluble proteases or other PSII proteins, for example, CP43 (Salter et al., 1992). Additionally, as can be seen in Figure 8, the observed inhibition is not due to a nonspecific effect, perhaps resulting from the presence of a foreign protein, since addition of control proteins such as carbonic anhydrase and lysozyme

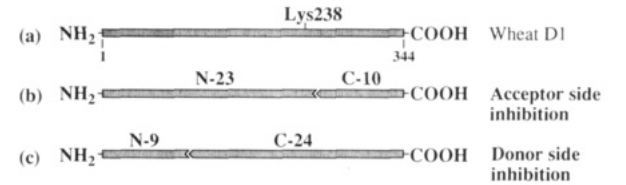

FIGURE 9: Schematic representation of the D1 protein showing the putative positions of the light-induced cleavages initiated by either acceptor side (b) or donor side (c) inhibition. Fragment sizes (in kDa) and origins are indicated; N, N-terminal; C, C-terminal. The position of cleavage by the exogenous lysine-specific protease, Lys-C, of wheat D1 protein is also indicated (a) [see Barbato et al. (1991) and De Las Rivas et al. (1992)].

did not inhibit the appearance of the 23-kDa fragment. More importantly, the inhibitory effect of SBTI on the production of the 23-kDa fragment did not occur if the inhibitor was denatured prior to use by heating to 80 °C for 5 min. It is also relevant to note that the addition of an equimolar concentration of SBTI to the reaction center suspension during the illumination period had no protective on the photoinduced loss of reaction center pigments or on electron-transfer activity (data not shown).

DISCUSSION

The work presented in this paper demonstrates that, under the appropriate conditions, photodamage of the isolated PSII reaction center can result in the appearance of the 23-kDa N-terminal fragment of the D1 protein (see Figure 9b). This finding, combined with other work (De Las Rivas et al., 1992; Ponticos et al., 1993; Friso et al., 1993), indicates that the light-induced degradation of the D1 protein observed in this minimal PSII complex follows two distinct pathways, involving either donor-side or acceptor-side inhibition (see Figure 9). When an electron acceptor is present, such as DBMIB or SiMo, the D1 protein degradation involves a cleavage leading to a 24-kDa fragment containing the C terminus of the mature protein and a corresponding 9-kDa N-terminal fragment (Barbato et al., 1991; De Las Rivas et al., 1992) (see Figure 9c). Under similar conditions, there also seems to be a secondary minor cleavage yielding N- and C-terminal fragments of about 16 kDa (Barbato et al., 1991; Friso et al., 1993). In both cases, the cleavages are thought to be due to inhibition on the donor side of PSII and located in the D1 polypeptide regions that form the hydrophilic loops joining putative transmembrane segments I to II and III to IV, respectively. The precise cleavage sites are not yet known, but the proteolysis is thought to be triggered by photochemical damage due to secondary oxidation processes resulting from the increased lifetime of $P680^+$ (Shipton & Barber, 1991). Indeed, photooxidation and degradation of β-carotene and chlorophyll are observed under these conditions (Telfer et al., 1990, 1991). It is possible that the damage due to these, or other, secondary oxidation events induces a conformational change in the reaction center structure which facilitates an autoproteolytic cleavage. Although the precise nature of the photochemical damage that induces this proposed conformational change is unclear, it does not require the presence of oxygen.

We have shown that oxygen must be present to observe the appearance of the light-induced 23-kDa N-terminal fragment of the D1 protein. This fragment was not observed in previous studies using isolated PSII reaction centers (Shipton & Barber, 1991; Chapman et al., 1989), probably because of the high pH used and the high irradiation doses given. As demonstrated here, when the pH was lowered to 6.0, a 23-kDa N-terminal

fragment was readily observed without the addition of exogenous electron donors or acceptors during the illumination of the reaction centers. In the absence of electron donors or acceptors, primary charge separation is followed by the recombination of the radical pair ($P680^+Pheo^-$), giving rise to the P680 triplet state (Takahashi et al., 1987; Durrant et al., 1990). Chlorophyll triplet states are known to be capable of interacting with oxygen to yield singlet oxygen ($^1O_2$).

$$^3P680 + {}^3O_2 \rightarrow P680 + {}^1O_2$$

Presumably, it is this reaction which underlies the photoinduced damage that triggers the degradation of the D1 protein to yield the 23-kDa N-terminal fragment. Previous studies indicate that singlet oxygen production by $^3P680$ brings about a selective irreversible bleaching of chlorophyll that absorbs at 680 nm (Telfer et al., 1990, 1991). These chlorophylls may well constitute P680 and could be responsible for generating singlet oxygen which has been detected directly in suspensions of isolated PSII reaction centers (Macpherson et al., 1993). Thus, it is possible that the selective destruction of the P680 chlorophylls by singlet oxygen induces a conformational change which is different from that associated with donor-side photoinhibition and allows a proteolytic event to occur in the loop between transmembrane segments IV and V on the acceptor side.

In the absence of amino acid sequence data, it is reasonable to assume that the 23-kDa N-terminal fragment is identical to the 23.5-kDa D1 protein degradation product detected *in vivo* by Greenberg et al. (1987). There is evidence (Shipton et al., 1990; Virgin et al., 1991; De Las Rivas et al., 1992) that the generation of this fragment involves a cleavage on the C-terminal side of residue 238 (see Figure 9c). The details of the proposed conformational change which leads to this cleavage have yet to be elucidated. Fourier transform infrared spectroscopy (He et al., 1991) has indicated that, when exposed to photoinhibitory light, the isolated PSII reaction center, in the absence of any additives, undergoes structural changes indicative of a conversion of α-helices into β-sheet. It is possible that the appearance of the 41-kDa D1–cyt *b559* adduct is in some way a reflection of this conformational change and is a prerequisite of the cleavage process. The photoinduced appearance of a band around 41 kDa has been observed under a number of different conditions (Shipton & Barber, 1991; De Las Rivas et al., 1992; Virgin et al., 1990). In the work presented here, the 41-kDa band seems to be related to the generation of the 23-kDa N-terminal fragment with a hint that it may be a precursor. For example induction of the 41-kDa band seems to precede the appearance of the 23-kDa fragment (see Figure 2a), and its formation is not inhibited by SBTI (see Figure 7). However, the immunologically derived data do not allow rigorous quantitative analysis of the relationship between the degradation of the D1 protein and the appearance of the 41-kDa D1–cyt *b559* adduct and 23-kDa breakdown fragment. Part of the reason for this is that the 23-kDa fragments seems to be unstable and does not accumulate to high levels (Ponticos et al., 1993). In addition, detailed quantification of the data is hindered by the existence of higher molecular weight bands due to D1/D2 aggregation. The level of this aggregated state is variable and occurs in untreated reaction centers as well as reaction centers exposed to light. It is difficult to judge from the western blotting techniques whether the level of the aggregated state changes after illumination, although, as Figure 8 shows, this treatment may induce D1/D2 aggregates of >60 kDa.

Evidence for the proteolytic nature of the cleavage comes from the temperature sensitivity of the production of the 23-kDa fragment and, more convincingly, from the action of the serine-type protease inhibitor SBTI. A similar conclusion has been reached from photoinhibitory studies using isolated PSII core complexes (Salter et al., 1992; Virgin et al., 1991). Our titration of inhibition by SBTI showed a 1:1 stoichiometry with the reaction center, which strongly suggests that the enzymatic activity is an inherent property of the isolated complex and does not involve any minor contaminants. Moreover, we have observed that the photoinduced formation of the 23-kDa N-terminal fragment is not dependent that the photoinduced formation of the 23-kDa N-terminal fragment is not dependent on the concentration of reaction centers (data not shown) and therefore suggests a cleavage mechanism which is intrinsic to each reaction center. The inability of SBTI to inhibit the photoinduced shift in the molecular mass of the D1 protein, as well as the appearance of the 41-kDa adduct, suggests that these changes probably do not involve any proteolytic activity and are likely to reflect events occurring prior to the enzymatic cleavage. Interestingly, the generation of the 24-kDa C-terminal fragment due to donor-side photoinhibition also involves a serine-type proteolytic activity (Shipton & Barber, 1992). However, since the two proposed cleavages are predicted to be on opposite sides of the complex, it is difficult to envisage a single active catalytic center involved in the production of both fragments.

Overall, we conclude that the 23-kDa N-terminal degradation fragment of the D1 protein can be observed when the PSII reaction center activity is poised toward radical pair recombination. On the other hand, when the lifetime of $P680^+$ is extended due to the efficient removal of electrons from reduced pheophytin, the 24-kDa C-terminal fragment accumulates. Potentially both events could occur *in vivo*, but to date experimental evidence only exists for both mechanisms in isolated PSII systems (Eckert et al., 1991; De Las Rivas et al., 1992; Friso et al., 1993). An EPR spin-polarized triplet signal of P680, due to radical pair recombination, has been observed in PSII-enriched membranes after exposure to photoinhibitory light treatment that led to the double reduction of Q_A, the primary quinone acceptor (Vass et al., 1992). This acceptor-side photoinhibition would, therefore, be expected to yield the 23-kDa N-terminal fragment and to depend on the presence of oxygen. Indeed, under anaerobic conditions the observed photoinactivation is reversible (Vass et al., 1992) and occurs without protein synthesis (Hundal et al., 1990). Donor-side photoinhibition may occur *in vivo* when the water splitting machinery is not working efficiently due, for example, to cold shock treatment (Wang et al., 1992). Alternatively, this mechanism may underlie the increased susceptibility of mutants to photoinhibitory light when modified on the donor side, for example, by deletion of the *psb*O gene encoding the 33-kDa manganese stabilizing protein (Mayes et al., 1991; Philbrick et al., 1991). These would be the conditions under which the 24-kDa C-terminal fragment would be expected to accumulate as a consequence of D1 protein degradation. Just how the balance between "acceptor"- and "donor"-side photoinhibition occurs *in vivo* has yet to be established, as has the nature of the D1 protein breakdown pattern under different enviromental conditions. Nevertheless, work with the isolated PSII reaction center presented in this paper demonstrates that both mechanisms exist within this relatively simple experimental system.

It has been reported that the D2 protein is also degraded *in vivo* under photoinhibitory conditions (Schuster et al., 1988),

albeit at a slower rate than that observed for D1. Photoinduced degradation of the D2 protein has been observed in isolated PSII preparations, namely oxygen-evolving cores (Virgin et al., 1990) and reaction centers (Shipton & Barber, 1991; Barbato et al. 1992b). Under the conditions required for production of the 23-kDa N-terminal D1 fragment, we found that the D2 protein was lost at a rate only slightly slower than that for D1. In contrast, the other subunits of the reaction center (α and β subunits of cyt *b559* and PsbI protein) were relatively stable (data not shown). Further work is needed to elucidate the origin of the breakdown products formed by degradation of the D2 protein under acceptor-side conditions.

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