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# On the Molecular Mechanism of Light-Induced D1 Protein Degradation in Photosystem II Core Particles†

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ABSTRACT: The mechanism of D1 protein degradation was investigated during photoinhibitory illumination of isolated photosystem II core preparations. The studies revealed that a proteolytic activity resides within the photosystem II core complex. A relationship between the inhibition of D1 protein degradation and the binding of the highly specific serine protease inhibitor diisopropyl fluorophosphate to isolated complexes of photosystem II was observed, evidence that this protease is of the serine type. Using radiolabeled inhibitor, it was shown that the binding site, representing the active serine of the catalytic site, is located on a 43-kDa polypeptide, probably the chlorophyll *a* protein CP43. The protease is apparently active in darkness, with the initiation of breakdown being dependent on high light-induced substrate activation. The proteolysis, which has an optimum at pH 7.5, gives rise to primary degradation fragments of 23 and 16 kDa. In addition, D1 protein fragments of 14, 13, and 10 kDa were identified. Experiments with phosphate-labeled D1 protein and sequence-specific antisera showed that the 23- and 16-kDa fragments originate from the N- and C-termini, respectively, suggesting a primary cleavage of the D1 protein at the outer thylakoid surface in the region between transmembrane helices D and E.

Photoinhibition of photosynthesis is targeted to photosystem II (PSII)[1] and seems to be an inevitable consequence of the complicated redox chemistry involved in light-driven water–plastoquinone oxidoreduction (Powles, 1984; Andersson & Styring, 1991; Barber & Andersson, 1992; Prasil et al., 1992). Photoinhibition leads not only to impairment of electron transport but also to irreversible damage to the reaction center of PSII. This damage is rectified by a repair mechanism involving high turnover of the D1 protein and to some extent

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[1] Abbreviations: Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; CP, chlorophyll protein; DFP, diisopropyl fluorophosphate; FTIR spectroscopy, Fourier transform infrared spectroscopy; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PSII, photosystem II; PVDF, poly(vinylidine difluoride); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, tricarboxylic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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the D2 protein. The molecular mechanisms for light-induced inactivation of PSII and D1 protein turnover are presently central topics in photosynthesis research. There is strong experimental evidence that inactivation of electron transport can be induced on both the acceptor and donor sides of PSII (Callahan et al., 1986; Andersson & Styring, 1991; Blubaugh et al., 1991; Eckert et al., 1991; Barber & Andersson, 1992; Prasil et al., 1992; Vass et al., 1992). Another problem relates to the mechanism of D1 protein turnover (Kyle, 1987; Mattoo et al., 1989), which involves the actual damaging events, the triggering for turnover, and the degradative steps (Andersson & Styring, 1991; Barber & Andersson, 1992; Prasil et al., 1992).

D1 protein degradation has been studied in vivo (Kyle, 1987; Mattoo et al., 1989; Prasil et al., 1992), but the reaction can also be observed in vitro (Andersson & Styring, 1991; Barber & Andersson, 1992). Degradation has been demonstrated after photoinhibitory illumination of isolated thylakoid membranes (Virgin et al., 1988; Richter et al, 1990), PSII membranes (Hundal et al., 1990; Kuhn & Böger, 1990), PSII core complexes (Virgin et al., 1990), and even isolated reaction center particles (Shipton & Barber, 1991; Andersson, 1992). The degradation reaction in vitro, which involves the production of reasonably stable D1 protein fragments, can be blocked by inhibitors acting against serine proteases (Virgin et al., 1991; Shipton & Barber, 1992). The degradation, which is temperature dependent (Aro et al., 1990; Richter et al., 1990; Shipton & Barber, 1992), is induced by photoinhibitory illumination but does not require light per se since it can proceed readily in total darkness (Aro et al., 1990). Taken together, these observations suggest that D1 protein degradation is due to proteolysis rather than to photosensitized cleavage reactions. Moreover, the results also suggest that the PSII complex harbors one or more endoproteolytic activities (Andersson & Barber, 1992), although the localization of proteolytically active sites to specific subunits remains to be determined.

The proteolytic cleavage sites within the D1 protein are still unknown. An interesting region is the stretch between helices D and E, exposed on the stromal side of the membrane (Trebst, 1986; Michel & Deisenhofer, 1988), which is rich in glutamate, serine, and threonine and therefore possesses a relatively high PEST-index score (Greenberg et al., 1987; Mattoo et al., 1989). A 23.5-kDa fragment has been observed in vivo (Greenberg et al., 1987), and proteolytic mapping suggested the cleavage site to be near amino acids 241–245 (Shipton et al., 1990). On the other hand, N-terminal sequencing of an in vitro produced fragment indicated a cleavage between amino acids 238 and 239 (Trebst & Depka, 1990). More recently, studies of D1 protein degradation in isolated PSII reaction centers have suggested a cleavage in the stretch between helices A and B on the lumenal side of the membrane (Barbato et al., 1991).

In this paper, we present further studies on the molecular pathway leading to D1 protein degradation in isolated subfractions of PSII following exposure to strong light. The results give a characterization of the conditions for optimal proteolysis. Moreover, by studying the binding of radioactively labeled diisopropyl fluorophosphate (DFP) to PSII core preparations, we were able to classify the protease as serine type. This labeling identifies a 43-kDa subunit, most likely the apoprotein of CP43, as a proteolytic subunit. An N-terminal 23-kDa fragment and a C-terminal 16-kDa fragment were shown to be primary proteolysis products of the D1 protein. This suggests that one initial cleavage occurs at the outer thylakoid surface within the sequence loop connecting transmembrane helices D and E.

## Materials and Methods

*Isolation and Subfractionation of Photosystem II Core Particles.* Thylakoid membranes were isolated from spinach leaves essentially as described in Andersson et al. (1976). PSII core particles were prepared from PSII-enriched membranes by solubilization with *n*-octyl β-D-glucopyranoside and differential centrifugation according to Ghanotakis et al. (1987).

In order to achieve further purification and to minimize contaminating polypeptides, PSII core particles were solubilized with 0.5% (w/v) dodecyl β-D-maltoside. The solubilized particles were subsequently applied to a Pharmacia Superose 12 gel filtration column connected to a Pharmacia FPLC system which had been previously equilibrated with 50 mM Mes, pH 6.0, 20 mM NaCl, and 0.05% dodecyl β-D-maltoside. The resulting PSII subcore particles were eluted from the column essentially as in Ghanotakis et al. (1987), collected by ultrafiltration (cutoff at 5 kDa), and finally suspended in 50 mM Mes, pH 6.0, 10 mM NaCl, and 0.4 M sucrose.

Fractionation of PSII subcore preparations into reaction center-, CP47-, and CP43-enriched fractions was done essentially as in Fotinou et al. (1991). The particles were solubilized with an equal volume of 20 mM Bis-Tris, pH 6.0, 4 M $LiClO_4$, and 2.5% dodecyl β-D-maltoside. After incubation, the mixture was desalted and loaded onto an anion-exchange column (Fast Flow Q, Pharmacia) previously equilibrated with 20 mM Bis-Tris, pH 6.0, 5 mM $LiClO_4$, and 0.05% dodecyl β-D-maltoside. Two of the main fractions, containing CP47 and reaction centers, respectively, were eluted with a gradient of $LiClO_4$. The fraction containing CP43 does not bind to the column and was contained in the void volume.

Chlorophyll determination was performed according to Arnon (1949).

*Photoinhibitory Treatment.* PSII particles were suspended at a concentration of 100 mg of chlorophyll·mL$^{-1}$ in 50 mM Mes, pH 6.0, 10 mM NaCl, and 0.4 M sucrose. Photoinhibitory illumination was performed in the range of 4000–8000 μE·m$^{-2}$·s$^{-1}$ using a projector lamp. The light output was modulated using neutral density and IR filters as necessary, and sample temperature was controlled in a thermostatic bath. During time-course experiments, samples were withdrawn and reactions stopped by immediately quenching the sample in liquid nitrogen and then placing the sample on ice in darkness. Illuminated core particles were collected either by centrifugation at up to 100000*g* or by ultrafiltration as described above.

*Phosphorylation of Thylakoid Proteins.* For $^{32}$P-labeling of specific proteins via the endogenous kinase activity (Bennett, 1983), the thylakoids were suspended in 50 mM Tricine, pH 7.6, 20 mM NaCl, 10 mM $MgCl_2$, and 100 mM sorbitol at a chlorophyll concentration of 0.4 mg·mL$^{-1}$. The phosphorylation medium also contained 0.4 mM ATP, including [γ-$^{32}$P]ATP [0.01 mCi·(mg of chlorophyll)$^{-1}$] and 10 mM NaF. Protein phosphorylation was induced by illumination at 500 μE·m$^{-2}$·s$^{-1}$ for 20 min at room temperature (Larsson et al., 1983). Phosphorylated PSII core particles were purified from these membranes as in Ghanotakis et al. (1987). Phosphorylated thylakoid membranes and PSII core particles were subjected to photoinhibitory illumination as described above.

*Polypeptide Analysis.* Polypeptides were separated on SDS–PAGE using the buffer system of Laemmli (1970) with gels either composed of a 12–22.5% polyacrylamide gradient in the presence of 4 M urea (Ljungberg et al., 1986) or composed of a linear 13% polyacrylamide concentration in the

presence of 6.5 M urea. Determination of the relative amount of D1 protein and fragments in a given sample was by quantitative western blotting. Proteins were electroblotted onto a PVDF membrane and immunodecorated with antisera according to Towbin et al. (1979) using $^{125}$I-labeled protein A for quantification. The antibodies used were raised either against the entire D1 protein or against a synthetic decapeptide corresponding to amino acids 333–342 of the spinach protein (Sayre et al., 1986).

Chlorophyll–protein complexes were resolved by mild SDS–PAGE according to Anderson et al. (1978). For the second dimension, the chlorophyll *a* bands corresponding to CP43 and CP47 were excised from the gel and reelectrophoresed under fully denaturing conditions as described in Larsson and Andersson (1985). Gels for fluorography were impregnated with Amplify (Amersham), dried, and autoradiographed. Quantification was performed by laser scanning densitometry.

*Diisopropyl Fluorophosphate Labeling.* Diisopropyl fluorophosphate (DFP) was obtained in 1,3-$^{3}$H and 1,3-$^{14}$C isotopic forms from NEN (DuPont) and as nonradioactive liquid from Merck. DFP stock solutions were diluted in propylene glycol to avoid decomposition. PSII core particles were labeled with DFP by incubation at 4 or 25 °C for up to 6 h in a buffer containing 50 mM sodium phosphate, pH 7.5, and 200 mM NaCl, after Maroux et al. (1971). Illumination, where indicated, was for 45 min at 5000 $\mu E \cdot m^{-2} \cdot s^{-1}$ either during or after incubation. Treated PSII core particles were collected by centrifugation or ultrafiltration as described above. Radioactive determination was performed following precipitation from 10% TCA onto Whatman GF disks and scintillation counting in Optiphase HiSafe scintillant. Identification of DFP-labeled polypeptides was done by fluorography as described above. Alternatively, bands were excised from polyacrylamide gels and dissolved in Solvable (DuPont), and the radioactivity was determined by scintillation counting in Formula 989 scintillant (DuPont) according to the manufacturer's instructions. Due to the extreme toxicity of DFP, all experiments involving this compound were performed under contained conditions.

## Results

*Characterization of D1 Protein Proteolysis.* No D1 protein degradation takes place at low temperature despite a pronounced photoinactivation, while after transfer to room temperature D1 protein degradation starts, in darkness, without any further loss of electron transport capacity (Aro et al., 1990). This effect offers an opportunity to experimentally separate the photoinactivation and protein degradative phases. Hence, by modification of the experimental conditions during this dark period, D1 protein proteolysis can be characterized in a direct manner. The pH dependence of the proteolytic reaction was analyzed using this experimental strategy (Figure 1). The D1 protein degradation showed a clear but relatively broad pH dependence. The proteolysis optimum was found at pH 7.5. The reaction was slightly stimulated by $Mg^{2+}$ ions, but not dependent upon their presence. The proteolytic activity was heat-stable and readily proceeded at temperatures of up to 50 °C.

*D1 Protein Degradation in Photosystem II Core Preparations.* PSII core particles can be isolated by differential centrifugation following detergent solubilization of PSII-enriched membranes (Ghanotakis et al., 1987). Such particles consist of the five polypeptides that make up the reaction center (Barber et al., 1987; Nanba & Satoh, 1987) plus the chlorophyll *a* binding proteins CP47 and CP43, intrinsic proteins

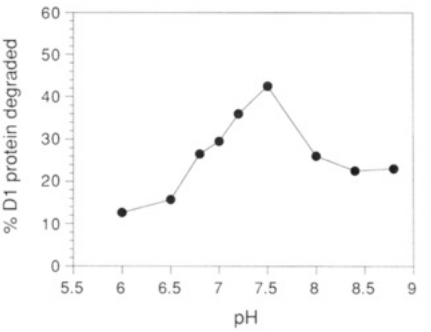


FIGURE 1: Graph demonstrating the pH optimum of D1 protein degradation. Thylakoids were illuminated at 2 °C as described (Aro et al., 1990) at pH 7.5. Degradation was then initiated by transferring samples, at the indicated pH, to room temperature and darkness. pH was manipulated by addition of 200 mM NaOH or HCl to the sample. The extent of D1 protein degradation after 1 h is shown, as measured by quantitative western blotting.

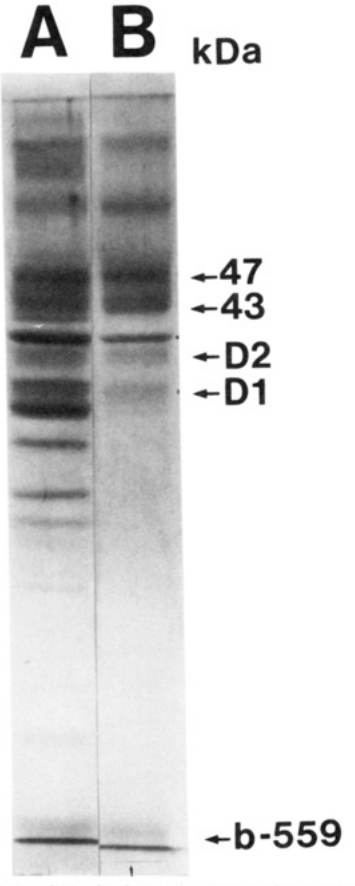


FIGURE 2: SDS–PAGE of PSII core (lane A) and subcore (lane B) particles separated on a 13% polyacrylamide gel containing 6.5 M urea in the buffer system of Laemmli (1970).

of 22 and 10 kDa, the extrinsic 33-kDa protein, and several low molecular weight proteins (Ghanotakis et al., 1987; Andersson & Styring, 1991). In addition, this preparation contains small nonstoichiometric amounts of polypeptides not belonging to the PSII core, which are detectable upon overloading during SDS–PAGE analysis (Figure 2). In a recent photoinhibition study, we showed that D1 protein degradation can occur in such a PSII core preparation and we suggested that a proteolytic subunit was an integral part of the PSII complex (Virgin et al., 1990). In the present study, we aimed to further investigate this intriguing possibility by reducing the polypeptide composition of the PSII particles in an attempt to minimize both the number of candidates for a proteolytic subunit and the risk of nonspecific protease contamination. Isolated PSII core particles were therefore subjected to further

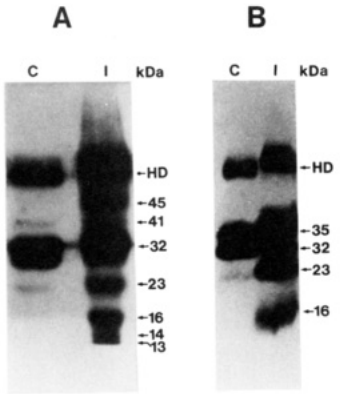


FIGURE 3: Immunoblot of isolated PSII core (A) and subcore (B) particles illuminated under high light. In both cases, lane C shows control PSII particles maintained in darkness, and lane I shows PSII particles illuminated for 30 min at 7000 $\mu E \cdot m^2 \cdot s^{-1}$, at 25 °C at a chlorophyll concentration of 100 $\mu g \cdot mL^{-1}$. The proteins were subsequently concentrated, separated by SDS–PAGE, electroblotted onto a PVDF membrane, and immunodecorated with anti-D1 protein antisera and $^{125}I$-protein A. HD, D1–D2 protein heterodimer. The photographs are deliberately overexposed in order to emphasize the observed fragments.

subfractionation by detergent solubilization and gel filtration chromatography (Ghanotakis et al., 1987). The resulting PSII subcore particles obtained showed a considerably simpler polypeptide composition (Figure 2). In particular, they lack the 22- and 10-kDa PSII subunits. Moreover, the low-abundancy contaminating polypeptides present in the original PSII core preparation are virtually absent.

Figure 3 shows an immunoblot demonstrating D1 protein degradation after photoinhibitory illumination at 20 °C of PSII core particles and purified subcore particles. In all samples, extensive degradation of the D1 protein occurred. Most degradation was seen for the 32-kDa monomer form of the D1 protein while only minor degradation of the heterodimer at 65 kDa and of the 35-kDa form of the protein occurred (Figure 3B). As determined by scanning densitometry, up to 80% of the monomer form of the protein can be lost, corresponding to approximately 50% of the total D1 protein (Figure 3B). In all cases, the degradation resulted in the appearance of two major fragments with apparent molecular masses of 23 and 16 kDa. Fragments in lower amounts could also be seen at 14, 13 (Figure 3A), and 10 kDa (not shown). Higher molecular mass fragments, attributed to the D1 fragment/D2 protein subheterodimer (Virgin et al., 1990), become evident at 41 and 45 kDa (Figure 3A). Notably, the recovery of D1 protein fragments can be very high compared to the amount of disappearing D1 protein, approaching 100% as judged from quantitative western blotting (Figure 3B). In PSII subcore particles kept as a dark control for the comparable period of time, the D1 protein was stable.

The possible involvement of a contaminating protease was also tested by performing dilution experiments in connection with photoinhibitory treatment of isolated PSII core particles. In more diluted samples of PSII core particles, a contaminating protease would cause less degradation of the D1 protein while proteolysis due to a component within the PSII complex would be independent of the concentration. For example, when PSII core particles were diluted from 0.4 mg of chlorophyll·$mL^{-1}$ to 0.1 mg of chlorophyll·$mL^{-1}$, there was no reduction in the rate of degradation. In fact, there was a slight increase in the extent of proteolysis, attributable to increased light flux through the diluted sample.

Taken together, these observations further strengthen the concept of an endoproteolytic activity within the PSII complex

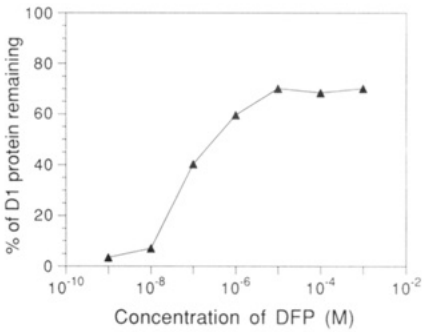


FIGURE 4: Isolated core particles (100 $\mu g$ of chlorophyll·$mL^{-1}$) were incubated for 4 h at 4 °C in the presence of the indicated amounts of DFP, recovered by centrifugation, washed, and then illuminated for 40 min at 8000 $\mu E$. Polypeptides were separated by SDS–PAGE, and the amount of D1 protein remaining was quantitated by western blotting. The amount on the figure is expressed as the percentage of a drak-maintained control sample.

that is able to degrade damaged D1 protein. Furthermore, these experiments demonstrate that the intrinsic 22- and 10-kDa polypeptides are not involved in the degradation of the D1 protein.

*Identification of a Proteolytic Subunit within Photosystem II.* Recent studies on various PSII preparations have shown that D1 protein degradation can be blocked by serine protease inhibitors but not by other classes of inhibitors (Virgin et al., 1991; Shipton & Barber, 1992). In order to conclusively classify a protease as belonging to the serine family, it is considered necessary to demonstrate inhibition by diisopropyl fluorophosphate (DFP) (Kraut, 1977). This inhibitor is taxonomic for serine proteases, binding to the active serine of the catalytic site. As illustrated in Figure 4, DFP gives a pronounced inhibition of D1 protein degradation when added to PSII core particles, in accordance with our previous study (Virgin et al., 1991). We wanted to demonstrate that this inhibition was indeed due to a covalent binding of DFP to the PSII core complex. Therefore, DFP at a concentration range of $1 \times 10^{-3}$ to $1 \times 10^{-9}$ M was added to PSII core particles at a final chlorophyll concentration of 100 $\mu g$ of chlorophyll·$mL^{-1}$. The treated particles were collected by centrifugation and washed before being subjected to photoinhibitory illumination. A varying reduction in the degree of inhibition of D1 protein degradation compared to untreated PSII core particles was obtained, the reduction depending on the concentration of inhibitor present during incubation (Figure 4). It can be seen that after an initial lag in the inhibitory binding, there is a quite linear relationship between DFP concentration and the inhibition of D1 protein proteolysis. The fact that the inhibition remains after washing indicates a binding between the complex and DFP. Inhibitor binding gives 75% reaction inhibition at $10^{-5}$ M DFP with half-inhibition at approximately $10^{-7}$ M. The binding of the inhibitor to the PSII core particles was investigated further using $^{14}C$- or $^{3}H$-radiolabeled DFP. Inhibitor present in the concentration range $1 \times 10^{-6}$–$1 \times 10^{-8}$ M was incubated with PSII core particles (100–200 $\mu g$ of chlorophyll) under both dark and photoinhibitory conditions. The binding of radiolabeled DFP was measured by scintillation counting after washing and collecting the particles by TCA precipitation. Quantification of the binding at $10^{-7}$ M suggests that on average ($n = 8$) each PSII core particle binds $0.52 \pm 0.38$ molecules of DFP. This value is dependent upon measurements of the number of chlorophyll molecules per isolated PSII complex, which vary from 70 to 90 depending upon the particular preparation.

The DFP binding occurred readily without preillumination of the PSII core particles, indicating that the serine of the

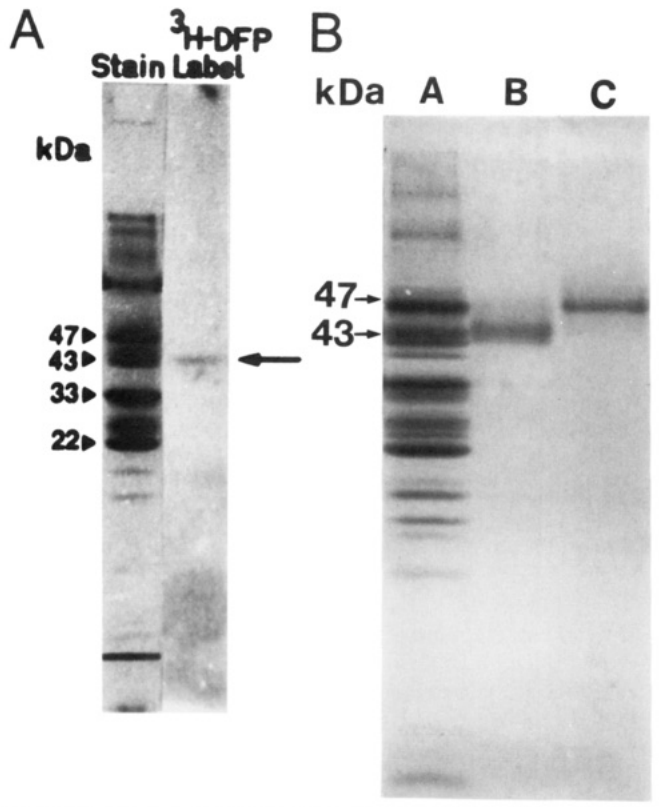


FIGURE 5: Panel A shows a Coomassie stain (left-hand lane) and fluorograph (right-hand lane) following SDS–PAGE of PSII core particles (100 $\mu$g of chlorophyll·mL$^{-1}$) incubated at 4 °C for 6 h in the presence of 1 $\mu$M [$^3$H]DFP as described. Panel B shows the polypeptides found after reelectrophoresis under fully denaturing conditions in the CP43 (lane B) and CP47 (lane C) chlorophyll–protein complexes which had been resolved by mild SDS–PAGE. The starting material is shown in lane A.

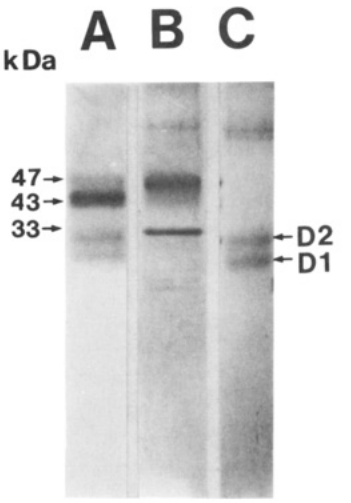


FIGURE 6: SDS–PAGE analysis of PSII fractions following detergent solubilization and ion-exchange chromatography of DFP-labeled PSII subcore particles. Lane A, CP43-enriched fraction; lane B, CP47-enriched fraction; lane C, PSII reaction center-enriched fraction.

catalytic site was accessible (active) in darkness. It is known that DFP binding to such an active site can be quenched by adding a substrate to the serine protease. In this particular case, involving D1 protein proteolysis as a consequence of photoinhibitory damage, competition experiments were performed by having DFP present during the strong illumination. The presence of photodamaged D1 protein, being the substrate for degradation, would make fewer activated serines available for DFP binding. These experiments showed that during photoinhibitory illumination there was an average 38% reduction in the degree of DFP labeling compared to controls kept for the same amount of time in darkness, consistent with the considerations made above.

The binding of radioactively labeled DFP to PSII, which is related to the inhibition of D1 protein degradation, offers a unique possibility to identify the responsible proteolytic subunit by fluorography after SDS–PAGE of labeled PSII particles. Figure 5A shows such an experiment using [$^3$H]DFP bound to PSII core particles. It can be clearly seen that the DFP has bound to one single polypeptide with an apparent molecular mass of 43 kDa. This labeled band comigrates with two closely-associated Coomassie-stainable polypeptides in the 43-kDa region. Calculations based upon the amount of TCA-precipitable input radioactivity and the intensity of the labeled band suggest that essentially all of the bound radioactivity is incorporated into this band. The same labeling result was observed using [$^{14}$C]DFP (not shown). No other bands became apparent even after very long exposure.

What is the identity of the labeled band? The most likely candidate is the 43-kDa apoprotein of the chlorophyll *a* binding complex, CP43, which gives rise to two distinct electrophoretic forms that migrate closely together (Ghanotakis et al., 1987). Alternatively, there could be a closely migrating polypeptide of unknown identity at 43 kDa. This problem of identity was addressed by first isolating both the CP43 and CP47 chlorophyll proteins by mild SDS–PAGE. The green bands were excised and reelectrophoresed together with isolated PSII core particles under fully denaturing conditions (Figure 5B). Since chlorophyll proteins have an electrophoretic migration different from fully denatured polypeptides (Anderson, 1980), no comigration of CP43 with non-chlorophyll binding polypeptides of molecular mass between 43 and 47 kDa is to be expected in the first-dimension gel. As revealed by reelectrophoresis (Figure 5B), the apoprotein corresponding to CP47 migrates well above the radiolabeled band. CP43 gives rise to both of the two bands in the 43-kDa region seen in Figure 5A. By excising the appropriate bands from preparative gels and scintillation counting the radioactivity therein, we could show that only the upper carried the radioactive label.

In still another attempt to identify the polypeptide associated with the DFP binding site, [$^{14}$C]DFP-labeled PSII subcore particles were subfractionated by detergent and chaotropic treatment followed by ion-exchange chromatography (Fotinou et al., 1990). This procedure yields three main fractions, these being enriched in the PSII reaction center, CP47, and CP43, respectively (Figure 6). After elution from the column, the fractions were subjected to TCA precipitation and scintillation counting. The analysis showed that 60% of the recovered precipitable radioactivity was found in the CP43 fraction (lane A) while 30% of the radioactivity was recovered in the CP47 (lane B) fraction and 10% in the reaction center fraction (lane C). Probably, the latter two figures are explained by contamination of the other fractions with CP43 (Figure 6).

*Primary Products of D1 Protein Proteolysis.* The possibility of obtaining relatively large amounts of D1 protein fragments after photoinhibitory illumination of PSII core particles should make it feasible to identify the cleavage sites within the D1 protein. Proteolysis of the D1 protein always gives rise to 23- and 16-kDa fragments and occasionally to 14-, 13-, and 10-kDa fragments (Figure 3). In order to determine which fragments are the primary products of the degradative reaction, aliquots were withdrawn at different time intervals during photoinhibitory illumination. It can be seen from the western blot analysis of Figure 7 that the 23- and 16-kDa fragments are the first to appear. Moreover, their simultaneous ap-

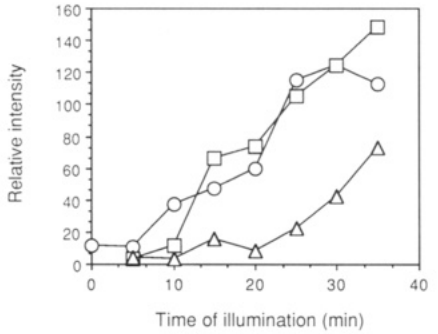


FIGURE 7: Isolated PSII core particles were illuminated at 25 °C, 6000 $\mu E \cdot m^2 \cdot s^{-1}$, at a chlorophyll concentration of 250 $\mu g$ of chlorophyll·$mL^{-1}$. Samples were withdrawn at the indicated times, quick-frozen in liquid nitrogen, and then stored on ice in darkness. Proteins were collected by ultracentrifugation at 60000$g$, separated by SDS–PAGE, electroblotted onto a PVDF membrane, and immunodecorated with anti-D1 protein antisera and $^{125}$I-protein A. The signal from each of the D1 protein fragments was quantitated and is expressed as optical density. The symbols used for the fragments are (○) 23, (□) 16, and (△) 10 kDa.

pearance during the course of light treatment is a strong indication that the 23- and 16-kDa fragments are primary proteolysis products. The 10-kDa fragment appears later after 20 min of illumination. This is also the case with the 14- and 13-kDa fragments, which were not obtained in the particular experiment presented in Figure 7. At present, it is not known whether these lower molecular weight fragments are derivatives from the two primary fragments or the products of a second, slower, endoproteolytic event acting directly on the D1 protein.

The two primary degradation fragments, when derived from PSII core particles, can be recovered together with the particles themselves by slow (6000$g$) centrifugation, suggesting that the 23- and 16-kDa fragments are still associated with the complex. This is consistent with the appearance of D1 protein fragments still bound to the D2 protein during SDS–PAGE analysis (Virgin et al., 1990; Figure 3).

N-Terminal sequence analysis of the 23- and 16-kDa fragments would allow direct identification of the proteolytic cleavage site on the D1 protein. However, several attempts to perform N-terminal sequencing on the two fragments have been unsuccessful, even when performed on relatively large amounts of material. Probably the fragments are N-terminally blocked. Whether this is a consequence of the experimental procedure or due to chemical modification of the D1 protein in connection with photoinhibitory damage, as suggested (Ohad et al., 1990), cannot be judged at present. In the absence of sequence information, we turned to some indirect analyses of the fragments. An antibody raised against a synthetic decapeptide corresponding to amino acids 333–342 of the C-terminal portion of the D1 protein (Sayre et al., 1986) was used to analyze the fragment pattern. As shown in the western blot of Figure 8, this site-specific antibody identified bands of 41, 32, and 16 kDa. No reaction was obtained with any other proteolysis product, including the 23-kDa fragment. This result strongly suggests that the 16-kDa fragment originates from the C-terminal portion of the D1 protein.

In another approach, we induced phosphorylation of thylakoid proteins prior to the photoinhibitory illumination. Under conditions when a particular kinase is active (Bennett, 1983), phosphorylation of LHCII and several PSII subunits, including the D1 and D2 proteins (Ikeuchi et al., 1987; Michel et al., 1988), occurs. This phosphorylation has been shown to occur at threonine residues at the N-terminal portion of the proteins (Michel et al., 1988). We performed photoinhibitory illumination and subsequent autoradiography analysis on

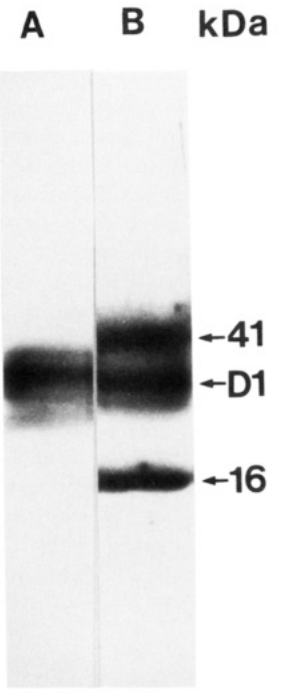


FIGURE 8: Isolated PSII core particles were illuminated for the indicated length of time at 7000 $\mu E \cdot m^2 \cdot s^{-1}$. Samples were collected by centrifugation, separated by SDS–PAGE, transferred to PVDF membranes, and immunodecorated. The antibody used was raised against a synthetic decapeptide corresponding to amino acids 333–342 of the C-terminus of the spinach D1 protein (Sayre et al., 1986). Lane A, control PSII core particles; lane B, PSII core particles illuminated for 30 min.

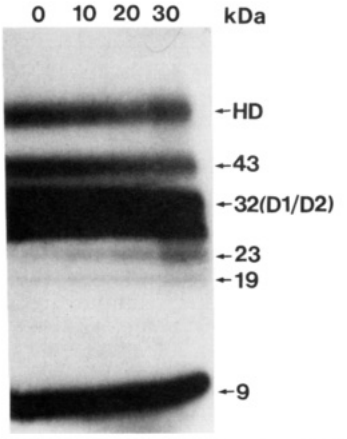


FIGURE 9: Thylakoid membranes were phosphorylated in the presence of [$^{32}$P]ATP, fractionated into core particles, illuminated at high light intensity, separated by SDS–PAGE, and autoradiographed. The $^{32}$P-phosphorylated PSII core particles were illuminated at 6000 $\mu E \cdot m^2 \cdot s^{-1}$ for the indicated times (minutes) and analyzed by SDS–PAGE and autoradiography.

thylakoid membranes which had previously been subjected to phosphorylation in the presence of [$^{32}$P]ATP, resulting in the N-terminus of the D1 protein becoming labeled. The analyses indicated a weak radioactive band in the 23-kDa region that accumulated in a light-dependent manner (not shown). However, these experiments were hampered by the fact that only very small amounts of D1 protein fragment can be recovered in thylakoids (Aro et al., 1990) and by the fact that the 23-kDa phosphorylated band migrates close to the bulky LHCII phosphoproteins. In subsequent experiments, the conditions were improved by first isolating PSII core particles from prephosphorylated thylakoid membranes. These PSII core particles, containing [$^{32}$P]phospho-D1 protein, were then subjected to photoinhibitory treatment as in the experiments

described above. Figure 9 shows a typical profile of PSII phosphoproteins, including incorporation of label into the D1 and D2 proteins. Notably, the figure also shows high light-induced accumulation of a 23-kDa phosphoprotein that is virtually absent in the control particles kept in darkness. No phosphate-labeled band appeared in the 10–16-kDa molecular mass region as a consequence of the photoinhibitory illumination. A high light-induced phosphorylated band at 19 kDa probably originates from a D2 fragment previously shown to have such a size (Virgin et al., 1990). The low amount of 23-kDa fragment observed, relative to that which might be expected from the degradation shown in Figure 3, is in accordance with recent studies (Aro et al., 1992; I. Carlberg, and B. Andersson, personal communication) showing that the phospho form of the D1 protein is a relatively poor substrate for the degradation reaction.

In conclusion, the photoinhibitory experiments with pre-phosphorylated PSII proteins and with the C-terminal site-specific antibody suggest that the 23- and 16-kDa fragments have N-terminal and C-terminal origins, respectively, within the D1 protein.

## Discussion

In this study, we have characterized an endoproteolytic activity within the PSII core complex that is essential for degradation of the D1 protein and hence of importance for the repair of photoinhibition. The degradative activity could conclusively be ascribed to a serine-type protease, due to the inhibitory effect of the taxonomic protease inhibitor diisopropyl fluorophosphate (DFP).

In studies on biodegradative reactions, the risk of contamination by unspecific proteases must always be considered. However, there are several experimental observations that strongly argue against such an explanation for the high degree of D1 protein proteolysis seen in the isolated PSII core and subcore preparations: (i) The same degree and pattern of proteolysis was seen even after extensive purification of PSII particles involving a combination of different separation methods; (ii) dilutions of the PSII core particles did not reduce the degree of D1 protein degradation, indicating a close association between substrate and enzyme; (iii) no proteolysis occurs without photoinhibitory illumination of the isolated particles, suggesting that only photodamaged and triggered D1 protein is a substrate for the protease; (iv) the inhibition of the degradation by DFP is correlated to covalent binding of this compound to a known PSII subunit; (v) at least one of the proteolysis products (23 kDa) corresponds in size to a D1 protein fragment previously observed in vivo (Greenberg et al., 1987).

The identification of a 43-kDa polypeptide in the PSII complex that covalently binds one molecule of DFP and consequently carries an activated serine is very interesting. Affinity labeling with DFP is highly specific—in the case of chymotrypsin, only 1 out of 28 serines is labeled (Kraut, 1977). The close relationship between binding of DFP to the PSII core particles and inhibition of the proteolysis reaction (Figure 4) gives support to the hypothesis that the 43-kDa subunit is indeed involved in the degradation of the D1 protein. All of our identification experiments point to the fact that DFP binding is targeted to CP43. A proteolytic activity as an integral part of a light-harvesting protein appears quite surprising, and further studies will be required on this matter. Studies on the D1 protein degradation pattern in PSII subpreparations completely lacking CP43, or mutants without this component, will be of particular importance.

The CP43 protein contains numerous serines, histidines, and aspartates (Alt et al., 1984), the amino acids that constitute the charge relay system in serine proteases (Kraut, 1977). However, since the three-dimensional structure of this protein is not known, any relevant predictions cannot be made. Multiple protein sequence analysis (not shown) suggests that of these amino acids 14 serines (as well as 13 histidines and 11 aspartates) are completely conserved in all known CP43 proteins but that none are found in motifs similar to those known to surround active-site serines. Thus, if CP43 carries a proteolytic activity, we are dealing with some new type of serine protease. However, this may not be very surprising since our knowledge about endogenous proteases, particularly those that are integral membrane proteins, is only in its infancy. Moreover, the serine protease mechanism per se is a common one, and has evolved separately on at least two occasions (Kraut, 1977). It is intriguing to note that a serine protease of 44 kDa has previously been identified as one of a number in spinach leaf tissue (Satoh & Fujii, 1982).

The binding between DFP and PSII core particles occurred readily in the dark, suggesting that in vitro the mechanism of proteolysis does not involve light-induced activation of the protease. Hence, the proteolysis is initiated through substrate activation, most likely through oxidative damage to the D1 protein in connection with the photoinhibitory event (Andersson & Styring, 1991; Barber & Andersson, 1992; Prasil et al., 1992; Vass et al., 1992). This triggering of D1 protein degradation (Aro et al., 1990) can be induced by damage originating from both the acceptor and donor sides of PSII and probably involves a conformational change within the photosystem. Such a change has been detected by FTIR spectroscopy of photoinhibited PSII reaction center particles (He et al., 1991). Similar changes are seen after certain detergent treatments of reaction center particles. It can therefore be speculated that changes, particularly conformational ones, to the D1 protein other than those induced by strong light could also turn the D1 protein into a substrate for the serine protease. In preliminary studies, we have observed D1 protein degradation in thylakoids heated to 40 °C in complete darkness (Sundby, Hundal, and Andersson, unpublished results).

Even if the serine protease seems to be present in its active form during darkness in vitro, it remains to be established if this is also the case in vivo. In a recent study using intact pumpkin leaves, it has been shown that a modification of the D1 protein, probably phosphorylation, precedes the degradation (Kettunen et al., 1991; Aro et al., 1992). This modification, designated D1*, is possibly the same as that suggested by Callahan et al. (1991). It was suggested (Kettunen et al., 1991; Aro et al., 1992) that this modification was essential for regulating the proteolytic process. Such regulation could be entirely at the substrate level although at the same time regulation at the enzyme level cannot be excluded, particularly since both the D1 protein and CP43 show the same phosphorylation behavior (Ikeuchi et al., 1987; Michel et al., 1988). On the other hand, recent studies (Aro et al., 1992; I. Carlberg and B. Andersson, personal communication) have shown that the phospho form of D1 is a relatively poor substrate for the proteolytic reaction in vitro (Figure 9). The significance of this finding, and its relationship to the situation in vivo, remains to be evaluated.

We have not been able by sequencing to directly demonstrate the origin of the 23- and 16-kDa proteolysis fragments. However, our experiments involving protein phosphorylation and a site-directed antibody indicate that the 23-kDa fragment is a product of the N-terminus of the D1 protein while the

16-kDa fragment is of C-terminal origin. In combination with the rapid and simultaneous formation of these two fragments, this suggests that the 23- and 16-kDa fragments represent the two products of a primary cleavage of the D1 protein. The sum of these two molecular masses gives a figure that overestimates the apparent size of the D1 protein (32 kDa). However, this can be explained by the relative nature of molecular mass estimation by SDS–PAGE. Notably, a 23.5-kDa fragment, but not a 16-kDa product, has been detected in *Spirodella* under in vivo conditions (Greenberg et al., 1987). This observation is consistent with the present 23-kDa fragment, and with the fact that this fragment is somewhat more stable than the 16-kDa fragment (not shown).

A primary cleavage yielding an N-terminal 23-kDa fragment and a C-terminal 16-kDa fragment is likely to occur at the loop exposed at the outer thylakoid surface between transmembrane helices D and E of the D1 protein, as has been previously suggested (Mattoo et al., 1989; Shipton et al., 1990; Trebst & Depka, 1990). This possibility is further supported by amino acid composition analyses of the C-terminal 16-kDa fragment after isolation by FPLC (Hagman and Virgin, unpublished results).

Our present results appear to be in contradiction with recent observations by Barber and co-workers on D1 protein degradation in photoinhibited PSII reaction center particles from pea and wheat (Barbato et al., 1991; Shipton & Barber, 1991, 1992). These particles, which supposedly contain CP43 only in residual amounts, can degrade the D1 protein in a light-dependent manner, and degradation fragments can be identified if an electron acceptor is present (Shipton & Barber, 1991). However, the fragment pattern appears to be different from that seen in the present study. Major degradation products are 24, 16, and 10 kDa where the two large ones are inferred to originate from the C-terminus while the 10-kDa fragment was suggested to be derived from the N-terminus (Barbato et al., 1991). Apart from phosphorylation and immunological studies, this assignment was based on the location of Lys-238 in the wheat D1 protein. This suggests a primary cleavage in the loop between transmembrane helices A and B (Barbato et al., 1991).

There could be several explanations for this discrepancy between the results obtained with PSII core and those obtained with reaction centers. Under our experimental conditions, we are dealing with acceptor side initiated photodamage (Andersson & Styring, 1991; Vass et al., 1992) while in the case of isolated PSII reaction center particles, with impaired water oxidation, there is donor side initiated photodamage (Shipton & Barber, 1991). This difference could be reflected in the subsequent triggering and proteolysis reactions. A more likely explanation may be that there is more than one endoprotease responsible for degrading the D1 protein. This would in fact seem to be necessary since the D1 protein is a transmembrane protein with loops exposed at both sites of the membrane, and hence in two different cellular compartments—the stromal space and the thylakoid lumen (Trebst, 1986; Michel & Deisenhofer, 1988). One single endoprotease would only have access to one side of the membrane. Taking the recent in vitro results on photoinhibition and D1 protein degradation together (Virgin et al., 1990, 1991; Barbato et al., 1991; Shipton & Barber, 1991, 1992; Barber & Andersson, 1992), it seems quite possible that there is one endoproteolytic activity associated with CP43 attacking the stromal side of the photodamaged D1 protein while another proteolytic activity more closely associated with the reaction center is attacking the lumenal side of the protein. Most likely the action of these two endoproteolytic activities is complemented by exoproteolytic activities for the complete degradation of the D1 protein. This possibility of multiple protease activities within the PSII complex and their sequential action on damaged D1 protein will require further studies.

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# Tissue Factor and Its Extracellular Soluble Domain: The Relationship between Intermolecular Association with Factor VIIa and Enzymatic Activity of the Complex†

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ABSTRACT: We find that the isolated, extracellular domain of tissue factor ($TF_{1-218}$; sTF) exhibits only 4% of the activity of wild-type transmembrane TF ($TF_{1-263}$) in an assay that measures the conversion of factor X to Xa by the TF:VIIa complex. Further, the activity of sTF is manifest only when vesicles consisting of phosphatidylserine and phosphatidylcholine (30/70 w/w) are present. To determine whether the decreased activity results from weakened affinity of sTF for VIIa, we studied their interaction using equilibrium ultracentrifugation, fluorescence anisotropy, and an activity titration. Ultracentrifugation of the sTF:VIIa complex established a stoichiometry of 1:1 and an upper limit of 1 nM for the equilibrium dissociation constant ($K_d$). This value is in agreement with titrations of dansyl-D-Phe-L-Phe-Arg chloromethyl ketone active site labeled VIIa (DF-VIIa) with sTF using dansyl fluorescence anisotropy as the observable. Pressure dissociation experiments were used to obtain quantitative values for the binding interaction. These experiments indicate that the $K_d$ for the interaction of sTF with DF-VIIa is 0.59 nM (25 °C). This value may be compared to a $K_d$ of 7.3 pM obtained by the same method for the interaction of DF-VIIa with $TF_{1-263}$ reconstituted into phosphatidylcholine vesicles. The molar volume change of association was found to be 63 and 117 mL $mol^{-1}$ for the interaction of DF-VIIa with sTF and $TF_{1-263}$, respectively. These binding data show that the sTF:VIIa complex is quantitatively and qualitatively different from the complex formed by $TF_{1-263}$ and VIIa.

The complexation of factor VIIa (VIIa)[1] with tissue factor (TF) is widely believed to be a critical step in the initiation of blood coagulation. As it occurs in nature, TF is a transmembrane glycoprotein consisting of an extracellular domain (residues 1-219), a single transmembrane domain (residues 220-242), and a cytoplasmic domain (residues 243-263) with

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[1] Abbreviations: VIIa, factor VIIa; X, factor X; sTF, soluble tissue factor; $TF_{1-263}$, full-length tissue factor; PS, phosphatidylserine; PC, phosphatidylcholine; FFRCk, D-Phe-L-Phe-Arg chloromethyl ketone; DF-VIIa, dansyl-D-Phe-L-Phe-Arg-labeled VIIa.

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