Degradation of the D1- and D2-Proteins of Photosystem II in Higher Plants Is Regulated by Reversible Phosphorylation†

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ABSTRACT: The effects of protein phosphorylation and dephosphorylation upon high-light-induced degradation of the photosystem II reaction center proteins D1 and D2 have been studied in isolated thylakoid membranes and photosystem II core complexes. The rate of photoinactivation of photosystem II electron transport is not affected by thylakoid membrane phosphorylation. However, the degradation rate of the D1-protein in its phosphorylated form is drastically reduced under conditions which induce either acceptor- or donor-side photoinhibition of photosystem II. The degradation rate of the D2-protein is also reduced following protein phosphorylation. The stability of the phosphorylated D1-protein is further increased under conditions of reduced phosphatase activity, suggesting that phosphorylated and damaged D1-protein has to be dephosphorylated prior to proteolytic degradation. Our results expand on experiments performed *in vivo*, which suggest that following photoinhibition the controlled repair of damaged photosystem II centers requires not only proteolytic enzymes but also kinase and phosphatase activities. It is suggested that the phosphorylation of the D1- and D2-proteins allows tight coordination of the degradation of damaged proteins with insertion of new copies of proteins into photosystem II.

The photosystem II (PSII)[1] reaction center consists of the D1- and D2-protein heterodimer, which by analogy with the L and M subunits of the reaction center in purple bacteria is thought to carry all the redox components required for the primary photoreactions [see Andersson and Styring (1991) and Vermaas et al. (1993)]. Light drives electron transport, but can also induce inactivation of PSII and damage to the D1-protein in a process called photoinhibition [see Prasil et al. (1992) and Aro et al. (1993)]. Consequently, the D1-protein undergoes rapid turnover (Kyle et al., 1984; Mattoo et al., 1984; Ohad et al., 1985) which is further accelerated by increasing irradiance. Degradation and turnover of the D2-protein also occur during photoinhibitory illumination, albeit to a lesser extent than that of the D1-protein (Schuster et al., 1988; Virgin et al., 1988; Trebst & Depka, 1990; Barbato et al., 1992). Photoinhibitory damage to the PSII reaction center is thought to be mediated via active oxygen species or by reactive endogenous radicals, like $P680^+$ and/or $tyrosine_z^+$ [see Andersson et al. (1992) and Barber and Andersson (1992)]. Damaged reaction center subunits are degraded and removed to allow for insertion of a new protein into the PSII complex in order to restore photosynthetic function [see Prasil et al. (1992) and Aro et al. (1993)]. The exact proteolytic system involved has so far not been identified, although the protease for the D1-protein has been shown to be of serine type (Virgin et al., 1991; Shipton & Barber, 1992). The protease is suggested, from *in vitro* studies, to be present in stoichiometric amounts in isolated PSII core complexes (Virgin et al., 1990; Salter et al., 1992a) and in reaction center particles (Shipton & Barber, 1991; De Las Rivas et al., 1993). Suggested protease candidates have been the CP43 chlorophyll *a* binding protein (Salter et al., 1992a) or the D1-protein itself (Virgin et al., 1990; Shipton & Barber, 1991). Alternatively, there are more recent suggestions of the involvement of a clp-type protease (Gong, 1994) or a nuclear-encoded proteolytic system (Bracht & Trebst, 1994).

There is accumulating evidence indicating that the rate of D1-protein degradation not only is determined by the extent of damage to PSII but also is under the control of some regulatory mechanism [see Aro et al., (1993)]. Previous experimental data indicate that a PSII complex preserves its damaged reaction center subunits until they can be replaced by newly synthesized protein copies (Kettunen et al., 1991; Schnettger et al., 1992). In addition, the degradation of a damaged D1-protein and the insertion of a new copy are proposed to be tightly coordinated (Adir et al., 1990; Aro et al., 1993). It has been suggested that damaged D1-protein is preserved due to phosphorylation and that this reflects a control mechanism for the repair of damaged PSII reaction centers in higher plants (Kettunen et al., 1991; Aro et al., 1992, 1993). Among the proteins of the PSII core complex, at least the D1 and D2 reaction center subunits, the CP43 of the inner chlorophyll *a* antennae (Michel et al., 1988; Marder et al., 1988) and also the *psbH* 9 kDa protein (Michel & Bennett, 1987) are posttranslationally phosphorylated. This phosphorylation occurs on threonines located at the N-

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[1] Abbreviations: chl, chlorophyll; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; P680, primary electron donor of PSII; PS, photosystem; Q_B, second plastoquinone electron acceptor of PSII; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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terminal regions of these proteins, which are at the outer thylakoid surface. However, unlike the situation for the reversible phosphorylation of the major light-harvesting chlorophyll *a/b* binding complex (LHCII), which is involved in antenna size modulations, the physiological significance of PSII phosphorylation is not known (Allen, 1992a). Phosphorylation of the D1-protein is clearly dependent on light intensity (Aro et al., 1992) and has been suggested to regulate the electron transport activity of PSII (Elich et al., 1993) or to influence the compactness of the PSII complex (Giardi et al., 1992).

In this paper, we provide evidence from *in vitro* studies that reversible phosphorylation of the D1-protein controls its degradation under various photoinhibitory conditions. The phosphorylated form of the D1-protein was found to be virtually resistant to degradation in thylakoid membranes isolated from both pumpkin and spinach leaves as well as in isolated PSII core complexes. The results further suggest that subsequent to dephosphorylation photodamaged D1-protein readily becomes a substrate for proteolysis. It is also demonstrated for the first time that degradation of the D2-protein is reduced following protein phosphorylation.

MATERIALS AND METHODS

Thylakoid Preparation. Spinach (*Spinacia oleracea* L. cv Medania) and pumpkin (*Cucurbita pepo* L. cv Jättiläismelooni) were grown at 23 °C with a 16 h photoperiod at 475 μmol of photons m^{-2} s^{-1}. Three week old fully expanded leaves, harvested after 1 h in the light, were used for preparations, if not otherwise stated.

Thylakoid membranes were isolated according to Andersson et al. (1976) and finally suspended in 100 mM sorbitol, 5 mM $MgCl_2$, 5 mM NaCl, and 50 mM Hepes−NaOH, pH 7.6 (storage buffer), at a concentration of 0.4 mg of chl mL^{-1}.

Preparation of PSII Core Complexes. PSII core complexes were isolated by octyl β-D-glucopyranoside incubation of BBY particles (Berthold et al., 1981) followed by sucrose gradient centrifugation (Ghanotakis et al., 1987). The isolated complexes were resuspended in 400 mM sucrose, 10 mM NaCl, 5 mM $CaCl_2$, and 50 mM Mes, pH 6.5, at 3−4 mg of chl mL^{-1} and stored at −80 °C until use.

Inactivation of the PSII Donor Side. Tris-washing of spinach thylakoid membranes was performed according to Jegerschöld et al. (1990) by resuspending the pelleted membranes in ice-cold 0.8 M Tris, pH 8.2. After incubation for 20 min under dim light, the membranes were washed twice with storage buffer.

Thylakoid Protein Phosphorylation and Dephosphorylation. Protein kinase activation was achieved by reduction of the plastoquinone pool, either by illumination of isolated thylakoid membranes at 250 μmol of photons m^{-2} s^{-1} (light-induced phosphorylation) or in darkness by incubation in the presence of 1 mg mL^{-1} NADPH and 10 μM ferredoxin (Sigma) (dark-induced phosphorylation). Protein phosphorylation was performed at 25 °C at 0.4 mg of chl mL^{-1} in storage buffer supplemented with 0.4 mM ATP and 100 μCi of [γ-^{32}P]ATP mL^{-1} (Amersham, 3000 Ci/mmol); 10 mM NaF was added to avoid dephosphorylation (Larsson et al., 1987). After 30 min of incubation, excess reactants (including all ATP) were removed by washing the thylakoid membranes twice with fresh storage buffer.

For dephosphorylation experiments, phosphorylated thylakoid membranes were pelleted and resuspended in 100 mM sorbitol, 5 mM $MgCl_2$, and 50 mM Hepes−NaOH, pH 7.6, at a concentration of 0.4 mg of chl mL^{-1}. Dephosphorylation was followed at 25 °C under low light (15 μmol of photons m^{-2} s^{-1}). To deplete Mg^{2+} ions, phosphorylated thylakoid membranes were washed in 100 mM sorbitol, 5 mM NaCl, 2 mM EDTA, and 50 mM Hepes−NaOH, pH 7.6, and suspended in storage buffer lacking $MgCl_2$.

Photoinhibitory Treatments. Thylakoids in storage buffer and PSII core complexes in resuspension buffer were illuminated by a 250 W slide projector at 2000 μmol of photons m^{-2} s^{-1} at 25 °C at a concentration of 0.4 mg of chl mL^{-1} (5 mm thick layer) for the indicated periods of time.

Oxygen Evolution Measurements. PSII oxygen evolution was measured as in Åkerlund et al. (1982) in a Clark-type oxygen electrode (Hansatech) at saturating light using phenyl-*p*-benzoquinone as an electron acceptor.

Polypeptide Analysis. SDS−PAGE was performed according to Laemmli (1970) using 15% acrylamide and 6 M urea in the separation gel. Resolved proteins were electrotransferred to a nylon membrane (Zeta-Probe, Bio-Rad) according to Towbin et al. (1979) and identified using antisera against the D1- and D2-proteins. The D1-protein antibody was raised in rabbits against a synthetic peptide corresponding to amino acids 234−242 of the D−E loop in *Synechocystis 6803* (Research Genetics, Huntsville, AL). This antibody showed similar sensitivity to unphosphorylated and phosphorylated D1-protein. The polyclonal D2-protein antibody was raised against the entire spinach D2-protein (a generous gift from W. Vermaas). For immunodetection, a chemiluminescence kit from Bio-Rad was used. Exposed films were scanned with a laser densitometer (LKB, Stockholm, Sweden). After immunodetection, the nylon membranes were washed overnight in 20 mM Tris, pH 7.5, 0.5 M NaCl, and 0.05% Tween-20 to remove chemiluminescent substrate and antibodies. [γ-^{32}P]ATP-labeled D1- and D2-proteins were detected by exposing the dried nylon membranes to phosphor screens (Bio-Rad). After 12−24 h exposure, phosphor screens were scanned with a Bio-Rad GS-250 Molecular Imager and analyzed with Phosphor Analyst software (Bio-Rad).

Kinetic Analysis. The rate constants for D1- and D2-protein degradation were obtained by fitting the data to an equation describing two sequential first-order reactions (Tyystjärvi et al., 1994) using FigP-software (Biosoft, Cambridge, U.K). The rate constant for the first reaction was obtained by fitting the data of photoinactivation of PSII oxygen evolution to a first-order equation, in order to take into account the observation that D1-protein degradation occurs in photoinactivated PSII centers (Tyystjärvi et al., 1994). Individual experiments were repeated at least 3 times. However, due to the involvement of at least three different enzyme activities (kinase, phosphatase, and protease), some variation between preparations could not be avoided, although the general trends were always reproducible.

RESULTS

On the Relationship between the Phospho D1-Protein and D1.* Isolated pumpkin thylakoids were phosphorylated in the light and then subjected to SDS−PAGE followed by

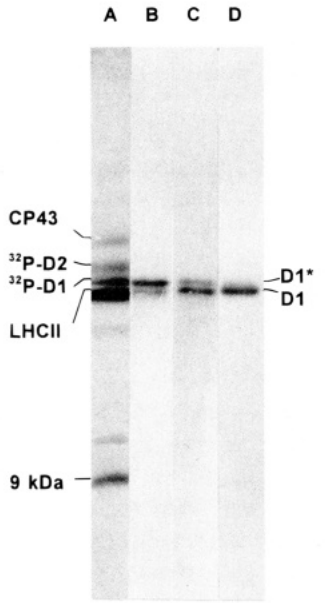

FIGURE 1: Effect of protein phosphorylation on the electrophoretic migration of the D1-protein as demonstrated by autoradiography (lane A) and immunoblotting (lanes B, C, and D). After light-induced protein phosphorylation in pumpkin thylakoids in the presence of [γ-^{32}P]ATP (lanes A and B), thylakoids were allowed to dephosphorylate under low-light conditions (15 μmol of photons m^{-2} s^{-1}) for 60 min (lane C). Lane D represents unphosphorylated thylakoid membranes isolated from dark-adapted leaves.

immunoblotting and autoradiography analyses (Figure 1). In control thylakoids, in which protein phosphorylation had not been induced, there is one major polypeptide recognized by the antibody against the D1-protein (Figure 1, lane D). After protein phosphorylation *in vitro*, another form of the D1-protein appears which has a somewhat slower electrophoretic migration (Figure 1, lane B), as judged by immunoblotting, in accordance with Aro et al. (1992) and Elich et al. (1992). This new band (designated D1*) is formed at the expense of the basic form of the D1-protein. Replacing ATP by GTP did not lead to any formation of D1*. Similar results were obtained for spinach thylakoids after phosphorylation had been performed in the dark in the presence of NADPH and ferredoxin (not shown).

When the phosphorylation experiment was carried out in the presence of [γ-^{32}P]ATP, the major typical thylakoid phosphoproteins LHCII, CP43, and *psbH* 9 kDa protein as well as the D1- and D2-proteins could be visualized by autoradiography (Figure 1, lane A). The ^{32}P-labeled D1-protein appeared concomitantly with, and showed the same electrophoretic mobility as, the D1* form detected by immunoblotting (Figure 1, lane B). Moreover, dephosphorylation resulted in transformation of D1* back to the D1-protein form (Figure 1, lane C). Taken together, these observations corroborate previous observations (Aro et al., 1992; Elich et al., 1992) based upon *in vivo* analyses that the D1* is the phosphorylated form of the D1-protein.

Quantifying the ratio between the two immunologically detected forms of the D1-protein shows that as much as 80% of the protein can be transformed *in vitro* into the D1* form in pumpkin thylakoids. For spinach thylakoids, we typically found 50% of the protein in the D1* form following phosphorylation.

Photoinactivation of PSII Oxygen Evolution in Phosphorylated and Unphosphorylated Thylakoid Membranes. The

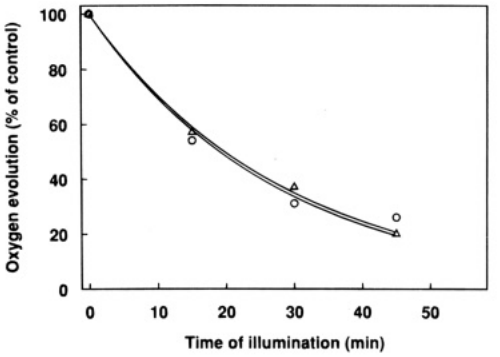

FIGURE 2: Inhibition of PSII oxygen evolution during acceptor-side photoinhibition at 2000 μmol of photons m^{-2} s^{-1} in unphosphorylated (○) and phosphorylated (△) spinach thylakoid membranes. Oxygen evolution activity of thylakoid membranes before and after phosphorylation was similar [160 μmol of O_2 (mg of chl)$^{-1}$ h^{-1}]. Data obtained were fitted to a first-order equation. The rate constants for photoinactivation, k_{pi}, were 35 ($\times 10^{-3}$ min^{-1}) for unphosphorylated and 37 ($\times 10^{-3}$ min^{-1}) for phosphorylated thylakoids.

time course of photoinactivation of PSII oxygen evolution in phosphorylated and unphosphorylated spinach thylakoid membranes during illumination at 2000 μmol of photons m^{-2} s^{-1} is shown in Figure 2. Notably, the PSII activity of both phosphorylated and unphosphorylated thylakoid membranes was equally susceptible to high-light-induced acceptor-side photoinactivation. The rate constant for photoinactivation of PSII, obtained by fitting the data to a first-order equation, was 36×10^{-3} min^{-1} for both unphosphorylated and phosphorylated thylakoid membranes (Figure 2).

Impairment of D1-Protein Degradation by Protein Phosphorylation in Isolated Thylakoid Membranes during Acceptor-Side Photoinhibition. Pumpkin and spinach thylakoids were isolated and phosphorylated *in vitro* in the presence of [γ-^{32}P]ATP and subsequently subjected to photoinhibitory illumination at 2000 μmol of photons m^{-2} s^{-1}. The time course for the disappearance of the phosphorylated and unphosphorylated D1-protein was compared by immunoblotting and autoradiography (Figure 3). In the spinach thylakoids, the unphosphorylated form of the D1-protein, measured by immunoblotting, decreased rapidly (Figure 3, open circles). The rate constant, k_{deg}, for D1-protein degradation was 36×10^{-3} min^{-1}. After 100 min of strong illumination, only 20% of unphosphorylated D1-protein remained. This rate of D1-protein disappearance is similar to that seen earlier in unphosphorylated control thylakoids from spinach (Virgin et al., 1990). In contrast, the loss of the D1* form of the protein in the same thylakoid sample was significantly slower ($k_{deg} = 10 \times 10^{-3}$ min^{-1}) (Figure 3, closed circles). Even after prolonged strong illumination, 50% of D1* remained. Moreover, the ^{32}P-labeled D1-protein, judged by autoradiography, showed the same slow disappearance, in agreement with a relation of D1* with phosphorylated D1-protein ($k_{deg} = 14 \times 10^{-3}$ min^{-1}) (Figure 3, open triangles).

Phosphorylated D1-protein also degraded more slowly than the unphosphorylated form in pumpkin thylakoid membranes. The degradation rates of the D1* and ^{32}P-labeled D1-protein under photoinhibitory conditions were even more reduced than in the spinach thylakoids (Table 1).

D1-Protein Degradation and Dephosphorylation. Strictly, the measurements described above on the loss of D1* or

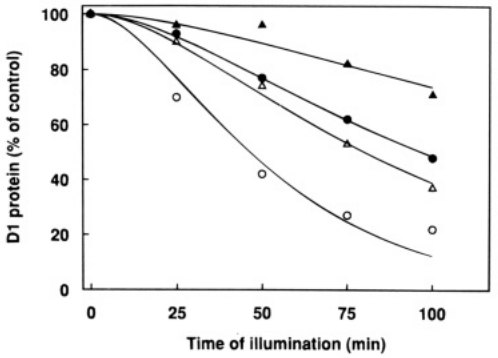

FIGURE 3: Degradation of the D1-protein in its phosphorylated and unphosphorylated form during photoinhibitory illumination of spinach thylakoid membranes. Thylakoids were phosphorylated by dark-induced phosphorylation in the presence of [γ-^{32}P]ATP, washed twice, and subjected to photoinhibitory illumination at 2000 μmol of photons m^{-2} s^{-1} for the indicated periods of time. Disappearance of the D1-protein (○) and D1* (●) was followed by immunoblotting. The loss of ^{32}P-labeled D1-protein in the absence (△) or in the presence of cold ATP (▲) was followed by autoradiography. Curves were obtained by fitting the data to an equation describing two sequential first-order reactions. Data points are means of three independent experiments. Standard error of the mean varied from 1 to 14%.

Table 1: Rate Constants (k_{deg}) for the Disappearance of Various Forms of the D1-Protein in Spinach and Pumpkin Thylakoid Membranes under High-Light Illumination[a]

D1-protein form	addition during illumination	$k_{deg} \times 10^{-3}$ min^{-1} [b]	
		spinach	pumpkin
D1	none	36	28
D1*	none	10	6.3
^{32}P-D1	none	14	7.6
^{32}P-D1	+cold ATP	4.2	3.9

[a] Thylakoid membranes were phosphorylated with [γ-^{32}P]ATP and washed twice, and subsequently the loss of unphosphorylated and phosphorylated D1-protein was followed in the absence and presence of cold 0.4 mM ATP. D1 and D1* were measured by immunoblotting and ^{32}P-D1 by autoradiography. Values were obtained by fitting the data to an equation describing two sequential first order reactions. [b] Rate constants for spinach were obtained from the traces shown in Figure 3. Values for pumpkin were obtained from similar experiments.

loss of ^{32}P-labeled D1-protein do not discriminate between the degradation and dephosphorylation. Experiments were therefore designed to follow the extent and rate of dephosphorylation of the D1-protein in pumpkin thylakoids. In particular, we aimed to find out which conditions can inhibit protein dephosphorylation without influencing PSII electron transport or D1-protein degradation. As can be seen in Figure 4A, dephosphorylation under low nonphotoinhibitory light conditions leads to transformation of D1* back into the unphosphorylated D1-protein form. Autoradiographic measurement (Figure 4B) shows that the half-time for D1-protein dephosphorylation was 35 min and that after 1 h of incubation as much as 60% was dephosphorylated. The rate constant for dephosphorylation (k_{deph}) was 20×10^{-3} min^{-1}. This dephosphorylation rate of the D1-protein could be reduced by the classical phosphatase inhibitor NaF ($k_{deph} = 2.8 \times 10^{-3}$ min^{-1}) or by removing Mg^{2+} from the incubation medium ($k_{deph} = 2.9 \times 10^{-3}$ min^{-1}) (Figure 4B). However, the use of these two effectors is not very appropriate in connection with photoinhibitory experiments, since NaF readily enhances inactivation of PSII oxygen evolution during high-light illumination and consequently

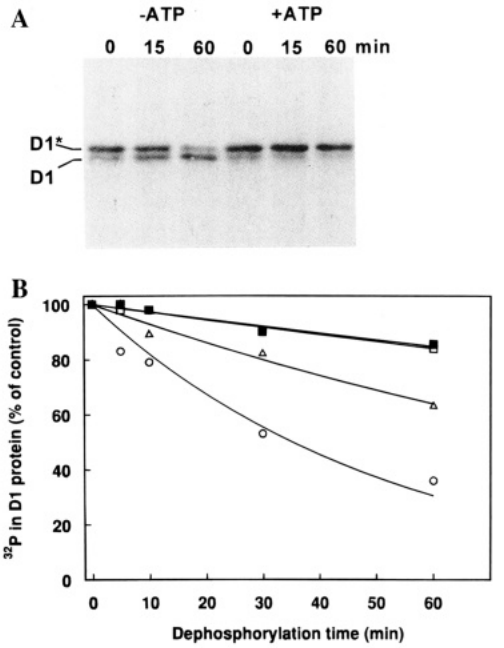

FIGURE 4: Dephosphorylation of the D1-protein in isolated pumpkin thylakoid membranes under low nonphotoinhibitory light conditions. Thylakoid membranes were phosphorylated in the presence of [γ-^{32}P]ATP, washed twice, and then allowed to dephosphorylate under the low light conditions (15 μmol of photons m^{-2} s^{-1}). (A) Dephosphorylation followed by immunoblotting for the indicated time periods in the presence or absence of 0.4 mM cold ATP. (B) Dephosphorylation followed by autoradiography: phosphorylated control (○), in the presence of 10 mM NaF (■) or 0.4 mM cold ATP (△), or after removing Mg^{2+}-ions (□). Data were fitted to first-order equations. The rate constants for dephosphorylation ($k_{deph} \times 10^{-3}$ min^{-1}) were 20, 2.8, 7.5, and 2.9, respectively. Data points are means of three independent experiments.

increases the rate of D1-protein degradation (not shown) and because D1-protein degradation optimally requires the presence of Mg^{2+} ions (Salter et al., 1992a).

Most phosphatases in cells are inhibited by millimolar concentrations of ATP [see Cohen (1989)]. Also, dephosphorylation of the D1-protein was impaired by ATP. As shown in Figure 4B (open triangles), the loss of [γ-^{32}P]ATP-labeled D1-protein under low-light conditions was over 2 times slower in the presence of cold 0.4 mM ATP ($k_{deph} = 7.5 \times 10^{-3}$ min^{-1}) than in the absence of ATP (Figure 4B, open circles). Moreover, at the same time, the transformation of the D1* back to the unphosphorylated D1-protein form was also inhibited (Figure 4A). GTP had only a minor effect on the dephosphorylation rate (not shown), demonstrating an ATP-specific effect rather than some secondary effect due to the lower concentration of available Mg^{2+} ions.

Consequently, cold ATP was included to reduce dephosphorylation during the photoinhibitory illumination of phosphorylated thylakoid membranes. [γ-^{32}P]ATP-phosphorylated thylakoid membranes, from which all excess radioactive ATP had been removed, were used. Autoradiographic measurements of the loss of phosphorylated D1-protein were not hampered by the presence of cold ATP, in contrast to immunological measurements of D1*, which were to some extent influenced by rephosphorylation in the presence of cold ATP (not shown). Notably, under photoinhibitory conditions, the loss of ^{32}P-labeled phosphorylated D1-protein was further reduced after addition of cold ATP (Figure 3, closed triangles, Table 1). The rate constant for the disappearance of ^{32}P-labeled D1-protein in spinach thylakoids

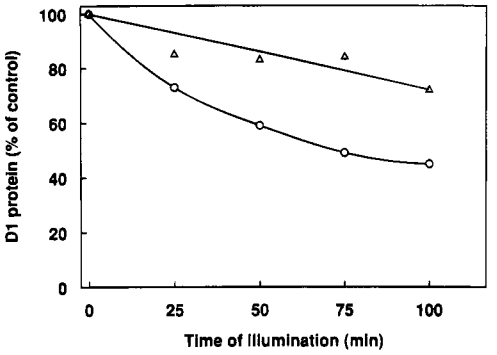

FIGURE 5: Degradation of the D1-protein and its phosphorylated form in isolated photosystem II core complexes from spinach. Photosystem II core complexes were isolated from [γ-^{32}P]ATP-labeled thylakoid membranes and then subjected to photoinhibitory illumination. The degradation of the D1-protein (○) and ^{32}P-labeled D1-protein (△) was followed by immunoblotting and autoradiography, respectively. Data points are means of two independent experiments.

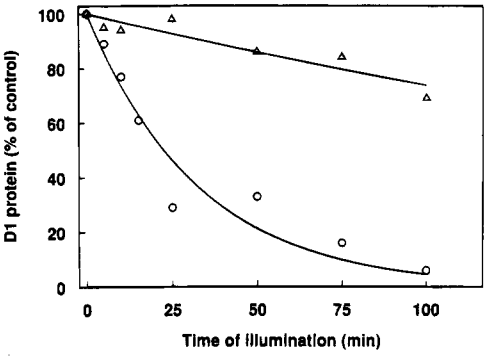

FIGURE 6: Degradation of the D1-protein and its phosphorylated form during donor-side-induced photoinactivation of spinach thylakoid membranes. The thylakoids were phosphorylated with [γ-^{32}P]-ATP in the dark, washed twice, and then subjected to alkaline Tris-washing followed by photoinhibitory illumination. The degradation of D1-protein (○) and the ^{32}P-labeled D1-protein (△) was analyzed by immunoblotting and autoradiography, respectively. Data points are means of three independent experiments.

was reduced from 14 × 10^{-3} min^{-1} to 4.2 × 10^{-3} min^{-1} (Table 1). Inhibition of D1-protein dephosphorylation by cold ATP also reduced the degradation rate of phosphorylated D1-protein in pumpkin thylakoids (from 7.6 x 10^{-3} min^{-1} to 3.9 × 10^{-3} min^{-1}, Table 1). We therefore conclude that the disappearance of phosphorylated D1-protein seen under photoinhibitory illumination in the absence of phosphatase inhibitors not only is a result of degradation but also is significantly influenced by dephosphorylation. Therefore, the uppermost curve in Figure 3 (closed triangles), where ATP was added to inhibit dephosphorylation, most likely represents the true degradation rate of phospho D1-protein under photoinhibitory illumination.

Effect of Phosphorylation on D1-Protein Degradation in Isolated PSII Core Complexes. In thylakoid membranes, there are a great number of phosphoproteins, and at present some 15−20 phosphorylated polypeptides have been detected [see Allen (1992a)]. Isolated thylakoids are therefore quite a complicated experimental system for mechanistic studies on the effect of phosphorylation on D1-protein degradation. Therefore, analyses were performed in a more simplified system using oxygen-evolving PSII core complexes, isolated from phosphorylated spinach thylakoid membranes. In such purified PSII core complexes, the influence of phosphatase activity would be expected to be minimal in contrast to thylakoids. It has previously been shown that degradation of the D1-protein can occur in such isolated PSII core complexes when subjected to strong illumination, in a reaction that is sensitive to various serine protease inhibitors (Virgin et al., 1991; Salter et al., 1992a).

Figure 5 shows the results from an experiment where spinach PSII core complexes, isolated from phosphorylated thylakoids, were subjected to photoinhibitory illumination. In these complexes, 17% of the D1-protein was found to be in the D1* form. The unphosphorylated D1-protein was rapidly degraded, in agreement with the previous studies on PSII core complexes (Virgin et al., 1991). In contrast, under identical conditions, in the same sample the disappearance of the ^{32}P-labeled D1-protein was extremely slow. The degradation pattern in the isolated PSII core complexes resembled very much the results obtained with isolated thylakoids when cold ATP had been added to reduce dephosphorylation (Figure 3, closed triangles).

Impairment of D1-Protein Degradation by Phosphorylation during Donor-Side-Induced Photoinhibition. The experiments with D1-protein degradation described in Figure 3 were carried out using thylakoids with an intact water-splitting system. Under such conditions, photoinhibition of PSII is thought to involve abnormal reduction and eventual loss of the quinone acceptors, triplet formation, and oxidative damage by singlet oxygen [see Andersson et al. (1992), Barber and Andersson (1992), and Aro et al. (1993)]. This acceptor-side photoinhibition has been suggested to lead to a primary cleavage around the interhelical loop between the two helices D and E of the D1-protein, which is exposed at the stromal side of the thylakoid membrane (Greenberg et al., 1987; Barber & Andersson, 1992). However, if thylakoids with an impaired electron donation to P680 are subjected to photoinhibitory illumination, the damage is thought to be induced by the formation of stable oxidizing radicals such as $P680^+$ and/or $tyrosine_z^+$ [see Barber and Andersson (1992)] and superoxide (Chen et al., 1992). This kind of donor-side-induced damage has been suggested to result in D1-protein cleavage primarily at the lumenal side of the thylakoid membrane, at the loop connecting the A and B helices (Barbato et al., 1991; De Las Rivas et al., 1992). We also investigated how this type of D1-protein degradation is affected by protein phosphorylation.

To this end, an experiment was designed in which phosphorylated spinach thylakoid membranes were treated by Tris-washing in order to destabilize the manganese cluster and impair the water-splitting system (Cheniae & Martin, 1978) prior to photoinhibitory illumination. As shown in Figure 6, there was a very rapid degradation of unphosphorylated D1-protein in thylakoid samples with an impaired donor-side, in agreement with previous results (Jegerschöld et al., 1990). However, under these donor-side photoinhibition conditions, the disappearance of the ^{32}P-labeled D1-protein was also very slow. After 75 min of strong illumination, as much as 70% of the phospho D1-protein remained, although only 15% remained for the unphosphorylated form of the protein. Thus, we conclude that under donor-side photoinhibitory conditions, as well as under acceptor-side conditions, the degradation of the phosphorylated D1-protein is reduced.

Effect of Phosphorylation on D2-Protein Degradation. The D2-protein of the PSII reaction center is also known to

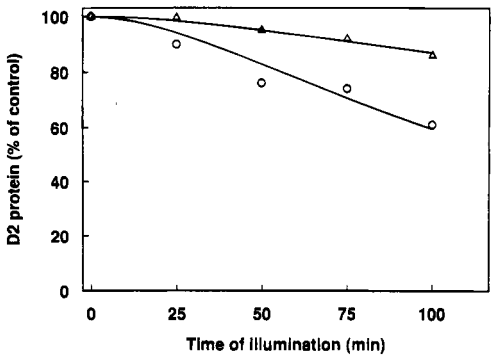

FIGURE 7: Degradation of the D2-protein and its phosphorylated form in spinach thylakoid membranes. Thylakoid membranes were phosphorylated in the dark in the presence of [γ-^{32}P]ATP, washed twice, and then subjected to photoinhibition under acceptor-side conditions. The disappearance of the D2-protein (○) (k_{deg} = 7.4 × 10^{-3} min^{-1}) and ^{32}P-labeled D2-protein (△) (k_{deg} = 1.9 × 10^{-3} min^{-1}) was analyzed by immunoblotting and autoradiography, respectively. For other details, see Figure 3.

undergo light-induced degradation [see Prasil et al. (1992)]. Although this protein is degraded to a lesser extent, its degradation shares many common features with the more well-characterized D1-protein degradation [see Andersson et al. (1994)]. Moreover, it is well established that the D2-protein, like the D1-protein, undergoes reversible phosphorylation [see Allen (1992a)]. The effect of phosphorylation on D2-protein degradation was therefore investigated by subjecting [γ-^{32}P]ATP-labeled spinach thylakoid membranes to strong illumination (Figure 7). The unphosphorylated form of the D2-protein showed a degradation rate (k_{deg} = 7.4 × 10^{-3} min^{-1}) leading to approximately 35% loss in 100 min, as judged by immunoblotting. The ^{32}P-labeled D2-protein, however, showed a considerably slower rate of degradation (k_{deg}=1.9 × 10^{-3} min^{-1}), and a clear loss of the phospho D2-protein could only be seen after prolonged photoinhibitory illumination. Similar observations were made during donor-side-induced photoinactivation (not shown). Thus, it can be concluded that the degradation of not only the D1-protein but also the D2-protein is influenced by reversible protein phosphorylation.

DISCUSSION

Turnover of the D1-protein is associated with the repair of damaged PSII reaction centers [see Prasil et al. (1992) and Aro et al. (1993)] although a regulatory turnover has also been considered [see Critchley (1994)]. Once a reaction center has been damaged under high-light stress, restoration of photosynthetic activity requires degradation of the damaged subunits and resynthesis and integration of new protein copies into the PSII complex. *In vivo*, net loss of the D1-protein has not generally been observed (Schuster et al., 1988; Kettunen et al., 1991; Schnettger et al., 1992), and under high-light conditions where photoinhibitory damage exceeds the rate of PSII repair, photoinhibited complexes with damaged D1-protein accumulate. This has been shown during chronic photoinhibition of *Dunaliella salina* (Smith et al., 1990) and in plants exposed to light intensities exceeding those of the growth conditions (Sundby et al., 1993; Aro et al., 1994). Such accumulation may be of physiological significance for avoiding secondary degradation of damaged and D1-protein-depleted PSII, since improperly assembled or ligated membrane complexes are known to be unstable and subjected to rapid proteolysis [see Goldberg 1992)].

The observations of light-induced formation of D1* in higher plant leaves (Kettunen et al., 1991; Elich et al., 1992, Rintamäki et al., 1995) and its identification as phospho D1-protein (Aro et al., 1992; Elich et al., 1992; Figure 1) suggest that reversible protein phosphorylation could be involved in D1-protein turnover. The present observations on both pumpkin and spinach thylakoids demonstrate that protein phosphorylation drastically reduces the D1-protein degradation during both acceptor- and donor-side photoinhibition conditions (Figures 3 and 6). It could even be argued that the phosphorylated form of the D1-protein may be completely stabilized against degradation except under very harsh or prolonged photoinhibitory illumination. This conclusion is based upon the observation that in samples with reduced phosphatase activity degradation of the phosphorylated D1-protein becomes very limited (Figures 3 and 5, Table 1). We therefore suggest that phosphorylated and damaged D1-protein is an intermediate stage in turnover that avoids excess D1-protein degradation in the absence of D1-protein synthesis. Only after dephosphorylation is the damaged D1-protein suggested to become susceptible to intrinsic proteolysis.

Our present finding, that the degradation rate of the phosphorylated form of the D2-protein is also reduced under photoinhibitory illumination, suggests a similar regulation for its degradation (Figure 7). As pointed out in a preliminary communication, D2-protein degradation shares many of the properties that have been ascribed to D1-protein degradation (Andersson et al., 1994).

In an earlier study by Harrison and Allen (1991), it was suggested that phosphorylation of PSII proteins protects against acceptor-side photoinhibition by suppressing free radical formation. However, this possibility is not very likely since in our experimental conditions, phosphorylation of thylakoid membranes modified neither the control rate of PSII oxygen evolution nor the susceptibility of PSII oxygen evolution to photoinactivation (Figure 2). Furthermore, the present results give additional support for a proteolytic cleavage of damaged D1-protein [see Andersson et al. (1994)]. This concept has been questioned by the ability to induce loss of D1-protein and appearance of degradation fragments *in vitro* simply by the addition of toxic oxygen species through the action of some chemical cleavage of peptide bonds (Mishra & Ghanotakis, 1994; Miyao, 1994). The present result, demonstrating that simply the introduction of a phosphate group at a N-terminal threonine of the D1-protein can specifically block its degradation under conditions where the unphosphorylated form of the D1-protein is readily degraded in the very same thylakoid or PSII core preparations, argues against photochemical cleavage. Our results therefore favor a model where the reactive oxygen species indeed induce oxidative damage to the D1-protein, but enzymatic activity is still required for the cleavage of the protein, in analogy with the removal of damaged proteins in other cells and tissues undergoing oxidative stress [see Wolff et al. (1986)]. The influence of phosphorylation on enzymatic degradation of proteins is known also from several other biological systems such as cytochrome P-450 (Eliasson et al., 1992) and Connexin-32 in plasma membranes from liver (Elvira et al., 1993).

It has also been speculated that a putative protease undergoing phosphorylation (Bracht & Trebst, 1994) or ATP-dependent clp-protease (Gong, 1994) could be involved in D1-protein degradation. In both cases, however, one would expect enhanced protein degradation in the presence of ATP, which is in contrast to our present results (Figure 3). Another possibility is that an intrinsic protease of the PSII core is inactivated by phosphorylation and this would coincide with reduced D1- and D2-protein degradation. In light of the results presented in this study, we favor a mechanism where protein phosphorylation renders D1-protein unavailable to proteolytic attack because phosphorylated D1- and D2-proteins have an altered conformation or otherwise unavailable recognition site. Studies on glycogen phosphorylase indicate that phosphorylated proteins can undergo conformational changes (Barford et al., 1991), and this concept has been introduced for thylakoid phosphoproteins by Allen (1992b). Moreover, it has been shown that phosphorylation of the D1-protein alters the herbicide binding affinity of the Q_B-pocket (Giardi, 1993), in line with a conformational change within the D1-protein. Indeed, there are a number of studies demonstrating that the occupancy of the Q_B-site by plastoquinone or added herbicides controls the rate of D1-protein turnover (Kyle et al., 1984; Mattoo et al., 1984; Trebst et al., 1988; Gong & Ohad, 1991; Jansen et al., 1993) by influencing the triggering for degradation (Salter et al., 1992b; Zer et al., 1994).

Furthermore, it was found that D1-protein degradation following donor-side induced photoinactivation was also reduced by protein phosphorylation (Figure 6). The primary cleavage of the D1-protein during such conditions is taking place at the lumenal side in the loop connecting helices A and B (Barbato et al., 1991; De Las Rivas et al., 1992), which is at the opposite side of the membrane with respect to the phosphorylation site at an N-terminal threonine at the stromal side. Consequently, a conformational change induced by the phosphorylation has to be transmitted through the thylakoid bilayer in order to influence degradation. This may be quite feasible, since perturbations at the donor side of PSII near the inner thylakoid surface readily affect the quinone acceptors which are located toward the outer side of the thylakoid membrane (Vass & Inoue, 1992).

In higher plants, the strict lateral compartmentation of the thylakoid membrane may require phosphorylation of D1-protein, since attachment of ribosomes and protein integration occur only in the stroma-exposed thylakoids (Wettern, 1986; Mattoo & Edelman, 1987) while PSII centers undergoing damage are located in the appressed regions (Aro et al., 1993). Furthermore, D1-protein phosphorylation has so far only been found in higher plants which possess a well-defined grana structure with a distinct organizational compartmentation into appressed and non-appressed thylakoid regions. In cyanobacteria (Kanervo et al., 1993), the green algae *Chlamydomonas reinhardtii* (de Vitry et al., 1991), and two moss species, *Marchantia* and *Ceratodon* (Rintamäki et al., 1995), all with no or limited formation of grana stacks, there is no clear evidence for D1-protein phosphorylation. However, our present finding that the phospho D2-protein is a poor substrate for degradation and that this protein is found to be phosphorylated also in lower eucaryotic photosynthetic organisms (de Vitry et al., 1991) makes it hard to make a general functional model for protein phosphorylation and degradation solely based upon thylakoid membrane differentiation. Recent studies even in the cyanobacterium *Synechocystis* 6803 (Komenda & Barber, 1995) suggest a coordination between degradation and synthesis of new D1-protein. This gives further support for the need of strong regulation of the repair process also in prokaryotic photosynthetic organisms although this may not involve protein phosphorylation.

In conclusion, we suggest that degradation of the D1- and D2-proteins of the PSII reaction center in higher plants is not only involving protease(s) but is also influenced by kinase(s) and phosphatase(s). Our understanding of this regulatory protein phosphorylation is largely hampered by the lack of detailed biochemical knowledge concerning the enzymes involved [see Allen (1992a)]. Their identity, precise location, and regulatory mechanisms are so far unknown, in particular for the phosphatase(s). Further studies are therefore required to elucidate the precise site and time for the phosphorylation/dephosphorylation of the D1-protein during the repair of PSII.

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