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Regulation of thylakoid protein phosphorylation in intact chloroplasts by the activity of kinases and phosphatases ☆

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Abstract

Regulation of thylakoid protein phosphorylation from saturating up to photoinhibitory light conditions was studied in intact chloroplasts with high rates of CO_2 fixation and functioning protein biosynthesis. Comparing steady-state level phosphorylation with the phosphorylation in the presence of the phosphatase inhibitor NaF, evidence was found that phosphorylation of light harvesting chlorophyll-protein complex II (LHC II) and Photosystem II (PS II) polypeptides was catalysed by at least two different kinase/phosphatase systems. Whereas the steady-state level of LHC II phosphorylation declined with increasing light intensity, the phosphorylation level of PS II polypeptides remained stable even at photoinhibitory conditions. Decrease of steady-state level of LHC II phosphorylation at higher light intensities was partially due to an inhibition of the phosphorylation reaction. Its inactivation was observed before any significant loss of PS II electron transfer activity occurred. Quenching analysis of chlorophyll fluorescence revealed that the high light inhibition of the LHC II phosphorylation reaction was caused by an increased membrane energetisation, and not by an oxidation of the plastoquinone pool. In contrast, the kinase activity responsible for the phosphorylation of the PS II polypeptides seemed to be exclusively under the redox control of the plastoquinone pool and was not influenced by membrane energetisation. Evidence was found that also the phosphatase activities specific for LHC II and PS II proteins were different. As indicated by the high turnover of phosphate groups bound to LHC II, the LHC II specific phosphatase showed a high activity. Its activity was stimulated at higher light intensities and determined to a main extent the steady state level of LHC II phosphorylation. The phosphatase specific for the PS II phosphoproteins showed almost no activity in the light as indicated by the absence of a phosphate group turnover in the light. Dephosphorylation of PS II could only be observed in the dark and in contrast to LHC II dephosphorylation could not be inhibited by NaF. Even the increased turnover of the D1 protein at higher light intensities, which was followed by the light-dependent incorporation of [^{14}C]leucine into the protein, did not accelerate the D1 protein phosphorylation. The steady-state level of PS II protein phosphorylation was therefore in principle determined by its kinase activity.

Keywords: Photosystem II; Phosphorylation; LHC II; Intact chloroplast; Protein kinase; Protein phosphatase

1. Introduction

Protein phosphorylation in eukaryotes has been shown to function as regulator of cellular processes. In plants, the most extensively studied phosphorylation phenomenon is associated with proteins of the photosynthetic membrane, especially those connected to PS II (for recent reviews see Refs. [1–3]). The main phosphorylated polypeptides belong to the LHC II protein family which forms the outer antenna of PS II. The other main group of phosphoproteins is located in the inner antenna and in the reaction centre core of PS II.

Abbreviations: F_0, yield of instantaneous fluorescence at open Photosystem II reaction centres in the dark; F_M, yield of maximum fluorescence when all reaction centres are closed by a saturating light flash; F_V, yield of variable fluorescence; LHC II, light harvesting chlorophyll-protein complex II; Mes, 4-morpholineethanesulfonic acid; PFD, photon flux density; PS II, Photosystem II; Q_A, primary quinone acceptor of Photosystem II; Q_B, secondary quinone acceptor of Photosystem II; q_P, photochemical quenching; q_N, non-photochemical quenching.

☆ Dedicated to Prof. Dr. Drs. h.c. A. Trebst on the occasion of his 65th birthday.

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To these polypeptides belong the 43 kDa, the D1 and the D2 protein and the 9 kDa psbH gene product. The D1 protein forms together with the D2 protein the PS II reaction centre core containing all redox components [4,5]. The D1 protein is characterised by a rapid light- dependent turnover [6], which seems to be involved in the process of photoinhibition [7,8]. Near to the PS II core, the CP 43 of the inner antenna and 9 kDa phosphoprotein are located. All thylakoid polypeptides except the 27 kDa LHC II polypeptide are phosphorylated at threonine residues facing the stroma side of the thylakoid membrane [2].

Most investigations regarding function and regulation of thylakoid protein phosphorylation have been concentrated on the LHC II protein family. LHC II phosphorylation is catalysed by a protein kinase which is under the redox control of the plastoquinone pool and is activated via the cytochrome b_6/f complex [9–11]. But there are also indications that cyclic electron flow and the demand for ATP might modulate LHC II phosphorylation [12–14]. As a result of phosphorylation, LHC II changes its conformation and cannot be bound any longer to the inner antenna of PS II [1,3]. In this way, energy transfer to PS II is reduced [2] and is redirected more to PS I [3].

Less is known about regulation and function of PS II protein phosphorylation. Their phosphorylation seems also to be coupled to the reduction of plastoquinone [1]. Recently, evidence has been provided that phosphorylation of the PS II reaction centre polypeptides is involved in regulation of PS II activity [15], maintaining its activity at high light intensities [16]. In addition, it has been assumed to play an important role in the rapid turnover of the D1 protein [17] increasing its stability against proteolytic cleavage [18].

In vitro, the steady-state level of LHC II and PS II protein phosphorylation is determined by the activity of their kinases [1,2]. Until now, three thylakoid protein kinases have been found with molecular masses of 25, 38 and 64 kDa [19,20]. According to Coughlan and Hind [20] 75–80% of thylakoid protein kinase activity is represented by the 64 kDa kinase in isolated thylakoids which is responsible for the phosphorylation of the LHC II polypeptides [10]. Whether it also catalyses the phosphorylation of the PS II belonging proteins has to be doubted. Even though it is under the redox control of the plastoquinone pool, the kinase activity responsible for the phosphorylation of the PS II reaction centre polypeptides differs in its sensitivity to sulfhydryl reagents and inhibitors of the LHC II specific kinase [2,9–11].

Kinetical studies have revealed the existence of at least four kinetically different types of phosphatase [21] associated with the thylakoid membrane [22,23]. They use phosphothreonine residues as a substrate whereas, phosphopeptides containing phosphoserine are unaffected [24]. In vitro studies have shown that the phosphatase activities are neither under light [23] nor under redox control [21]. The phosphorylation status of thylakoid proteins in isolated thylakoids is therefore determined mainly by the activity of the kinase.

Recent studies from Elich et al. [25] have shown that in vivo thylakoid phosphatase activity can also be stimulated in the light. Thus, thylakoid protein phosphorylation in vivo seems to be more complexly regulated than in vitro. From in vitro systems it is known that increase in light intensities results in an increase of thylakoid protein phosphorylation, especially of the LHC II, since the kinase activity is stimulated by the increase in the reduction level of the plastoquinone pool. In intact systems, like green algae or leaves of higher plants [26,27], the steady-state level of LHC II phosphorylation declined at high light conditions. Neither the regulation nor the function of this phenomenon is completely understood. It has been discussed that the LHC II kinase might be deactivated either by a decreased reduction state of the plastoquinone pool [26] or by an increase in ΔpH [27,28]. But, according to Schuster et al. [26], phosphatase activities might also be important in regulating the steady-state level of thylakoid proteins.

To address the problem of phosphorylation control by kinase and phosphatase activities, we studied the incorporation of [^{32}P]orthophosphate in intact chloroplast with high activities of CO_2 fixation. The *organello* system should possess regulatory mechanisms similar to those in intact plants. However, it has the advantage that substrates and inhibitors can be easily applied in defined concentrations, which is not possible in leaves of higher plants. Our results provide evidence that phosphorylation of PS II proteins and of LHC II is differently regulated. In contrast to the findings of Elich et al. [25], we found that the steady-state level of LHC II phosphorylation is controlled by both kinase and phosphatase activity due to a highly active phosphatase. With increasing light intensity, phosphatase activity becomes highly stimulated, resulting in a dephosphorylation of LHC II. The phosphatase specific for PS II polypeptides showed a very low activity in the light and therefore their level of light-dependent steady-state phosphorylation was only determined by the kinase activity. The significance of the PS II reaction centre phosphorylation for the D1 protein turnover is discussed.

2. Material and methods

2.1. Isolation of intact spinach chloroplasts

Freshly harvested spinach leaves from the greenhouse were preilluminated 30 min while floating on

cold water. The leaves were then shaken dry and sliced to small stripes without midrib parts. Isolation of intact chloroplast was performed according to Walker [30] with the following modifications. 15 g spinach were blended in a domestic blender (Sorvall Omni-Mixer 17106) for 5 s using maximum speed and 100 ml buffer containing 0.33 M sorbitol, 0.05 M Mes-KOH (pH 6.5), 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 2 mM EDTA (consistency of melting snow). Then the mixture was kept on ice and immediately centrifuged for 90 s at 6200 rpm in a Hettich Roto Silenta II centrifuge. The supernatant was poured off and the pellet was washed twice using ice-cold resuspension buffer containing 0.33 M sorbitol, 0.05 M Hepes-KOH (pH 7.6), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA. Then the pellet was resuspended in 1 ml resuspension buffer and stored on ice in the dark. Chlorophyll content was determined according to Arnon [29].

2.2. Determination of chloroplasts intactness / CO_2-dependent O_2 evolution

The intactness of the chloroplasts was measured in an oxygen electrode using modified resuspension buffer with an addition of 10 mM D,L-glyceraldehyde, 2.5 mM ferricyanide and 2.5 mM NH_4Cl at 20°C. The percentage of intactness was calculated from the relationship $(A - B)/A \times 100$ in which A is the rate of the osmotic shocked chloroplasts and B is the rate without osmotic shock. The CO_2-dependent O_2 evolution was determined using an assay buffer containing 0.33 M sorbitol, 0.05 M Hepes-KOH (pH 7.6), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.05 mM KH_2PO_4 and 10 mM $NaHCO_3$ at 20°C. As a result of this determination only intact chloroplast preparations were used with a minimum rate of 50 μmol O_2 (mg chlorophyll)$^{-1}$ h^{-1} oxygen evolution and an intactness above 80%.

2.3. Light-dependent phosphorylation of thylakoid proteins

Phosphorylation experiments were performed by incubating a total amount of 50 μg chlorophyll equivalent per 1 ml assay buffer in the presence of 40 μCi [^{32}P]orthophosphate. The incubation was carried out in glass tubes at 20°C using a water bath. For illumination Hedler de Luxe 2000 white light sources were used. To check phosphate group turnover ^{32}P-protein labelling was measured using defined incubation intervals after preillumination under identical light conditions. The dephosphorylation reaction of thylakoid phosphoproteins in the dark was observed after 20 min prelabelling phase in the light. Phosphorylation assay reactions were terminated via freezing 250 μl aliquots of assay mixture in liquid nitrogen adding 10 mM NaF to prevent protein phosphatase reactions.

2.4. D1 protein synthesis assay using [^{14}C]leucine polypeptide labelling

Incubation of intact chloroplast was carried out as described for the phosphorylation assay with the modification that [^{14}C]leucine (NEC-279E, NEN/Du Pont) was used instead of [^{32}P]orthophosphate. For incubation, a total amount of 185 kBq [^{14}C]leucine (11.5 GBq/mmol) per 1 ml assay mixture was added.

2.5. SDS-PAGE, immunoblotting and autoradiography

SDS-PAGE was performed as previously described [31] with the following modifications. Thawed assay aliquots were centrifuged immediately in an Eppendorf mircofuge for 4 min at 14000 rpm. Then the supernatant fluids were poured off and the pellet was solubilised in 5% SDS, 15% glycerol, 50 mM Tris (pH 6.8) and 2% mercaptoethanol at room temperature for 30 min. The polypeptides were separated by polyacrylamide gel (15%) electrophoresis according to Schägger et al. [32]. Then polypeptides were transferred to nitrocellulose (Schleicher & Schuell, BA 100, 200 μm) using a Bio-Rad Transblot chamber. Electroblotting was carried out for 3 h at 0.4 Å in a cold room. For autoradiography different Western blot preparations were performed. Immunodetection of D1, D2, CP 43 and LHC II was carried out according to Godde et al. [31]. For Coomassie-stained protein pattern Western blots were incubated in 1/10-fold stain solution for 2 min and destained for 2 h. After both types of preparation, nitrocellulose was air dried for 2 h. Radiolabelled polypeptides were visualised by autoradiography of Western blots using a Kodak X-Omatic exposure cassette and Fuji RX NIF film material. The autoradiograms were analysed using a LKB 2222-020 Ultrascan laser densitometer and GelScan XL 2.1 software. The preparation of the D1 protein accumulation assay samples was carried out in the same way. For autoradiography, Amersham film material Hyperfilm-βmax RPN was used and analysed with the same LKB equipment.

2.6. Fluorescence relaxation after a single-turnover flash

Chlorophyll fluorescence decay after a single-turnover flash was examined as previously described [31] with the following modifications. The light-treated intact chloroplast assay mixture was transferred to a Bachhofer DW 2/2 oxygen electrode and dark-adapted for 5 min. Then fluorescence decay measurements were carried out using PAM fluorometer 101, 103 and XST103 (Walz, Effeltrich, Germany) with the PAM 101 switch positions measuring light = 10/gain = 6. The decay kinetics were sampled using the DA 100 software. Final exponential analysis of fluorescence

decay was carried out with the software written by Volker Ebbert (see also Ref. [31]).

2.7. Saturating pulse-modulated chlorophyll fluorescence measurement with simultaneous O_2 evolution detection

Chlorophyll fluorescence measurement was carried out with the same equipment as mentioned before with the following modifications. Instead of an XST103 Xenon flash lamp, two Schott KL 1500 white light sources were used for actinic and saturating illumination. The measurements were performed with PAM fluorometer 101 switch settings of measuring light = 10/gain = 6. First F_0 and F_M were determined using a 5 min dark-adapted sample by a saturating light flash (500 ms duration/Schott KL 1500 setting = 5 full light). After a 1 min relaxation period in the dark q_N and q_P were measured according to Schreiber et al. [33] with little modification. Three actinic light PFDs were used (375, 630 and 1075 μmol m^{-2} s^{-1}) with saturating light flashes repeating every minute. For q_N and q_P calculation the nomenclature of Van Kooten and Snel [34] was used. Oxygen evolution was determined simultaneously as described before.

3. Results

3.1. Photosynthetic properties of intact spinach chloroplasts

To check how different photon flux densities affect PS II activity, intact chloroplasts were illuminated with increasing PFDs starting from 125 up to 5000 μmol m^{-2} s^{-1} for 30 min. As shown in Fig. 1 photosynthesis of intact chloroplast was completely light-saturated at 630 μmol m^{-2} s^{-1} represented by CO_2-dependent oxygen evolution (Fig. 1A). Above this light intensity the primary quinone acceptor of PS II remained completely reduced as indicated by the total loss of photochemical quenching of chlorophyll fluorescence, q_P. The increased quenching at higher light intensities was therefore completely due to the non-photochemical quench, q_N. Since q_N is determined to a great extent by the energy state of the thylakoid membrane [35], the experiment shows that pH increases at higher light intensities when photosynthesis in intact chloroplasts is already saturated. NaF at a concentration of 10 mM inhibited CO_2-dependent oxygen evolution to about 50% (data not shown). However, it had no effect on the variable fluorescence nor on the quenching parameters.

Electron flow within PS II was determined by the fluorescence yield after a single turnover flash which reflects the ability of PS II to reduce its primary quinone acceptor Q_A [35]. To oxidise Q_A^- and the plastoquinone pool the chloroplasts had been dark-adapted for 5 min prior the fluorescence yield measurements. Further dark-adaptation had no effect on the fluorescence yield. The fluorescence yield, F_V, was standardised to F_M. As shown in Fig. 2, illumination with 125 μmol m^{-2} s^{-1} had no effect on the ability of PS II to reduce Q_A. Starting with PFDs of 500 μmol m^{-2} s^{-1}, the ratio of F_V/F_M decreased with increasing PFDs. The highest loss of F_V/F_M was observed after 30 min illumination with 5000 μmol m^{-2} s^{-1}.

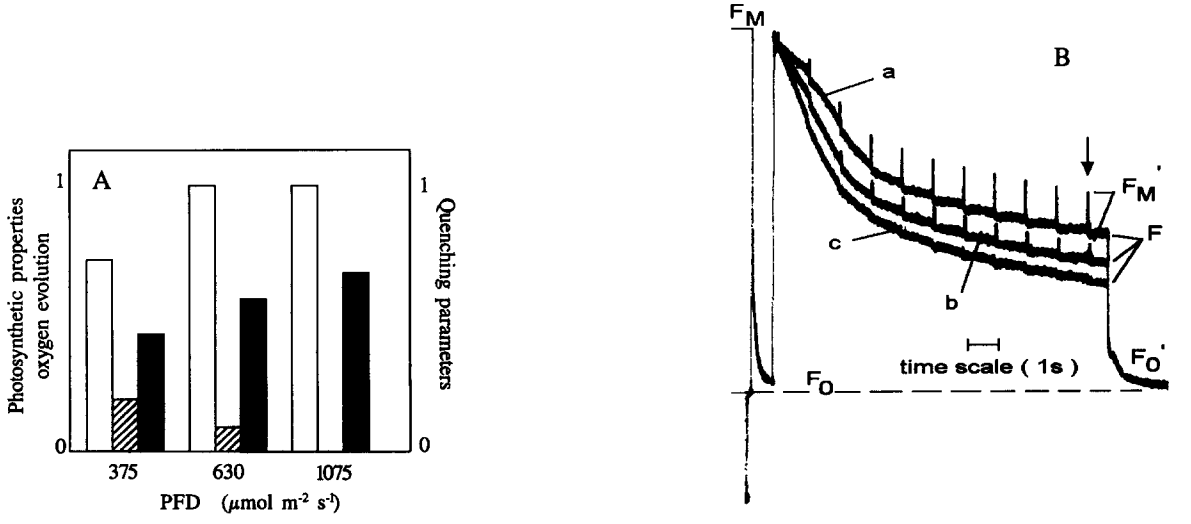

Fig. 1. Photosynthetic properties of intact spinach chloroplasts. (A) In intact chloroplasts CO_2-dependent oxygen evolution (□) is saturated at a PFD of 630 μmol m^{-2} s^{-1}. At higher light intensities an increase of q_N (■) and a decrease of q_P (▨) can be observed when CO_2-dependent oxygen evolution is already saturated. Maximum CO_2-dependent oxygen evolution was normalised to 1 (100%). (B) "Saturation pulse method" fluorescence measurement. Quenching analysis was carried out for PFDs of 375 (a), 630 (b), up to 1075 (c) μmol m^{-2} s^{-1} in intact chloroplasts. Determination of q_N and q_P took place at the time point marked by the arrow. At the beginning of fluorescence measurement a F_V/F_M ratio of 0.635 was determined.

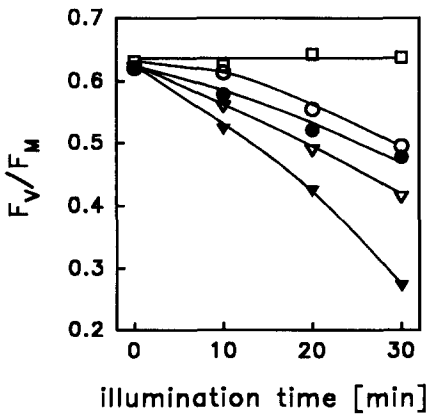

Fig. 2. Photoinactivation of Photosystem II in intact spinach chloroplasts measured by fluorescence decay after the indicated preillumination. With increasing PFDs of 125 (□), 500 (○), 2500 (●), 3750 (▽) and 5000 (▼) μmol m^{-2} s^{-1} the ratio of F_V/F_M, which represents PS II activity, decreases during illumination. The highest loss of F_V/F_M was observed after 30 min illumination at 5000 μmol m^{-2} s^{-1}.

After this time only 45% of full functional PS II centres were left.

The fluorescence decay after a single turnover flash is known to reflect the kinetics of Q_A^- reoxidation [35,36]. It decays in three exponential phases (Table 1). The rapid phase, Amp_f, with a half-life time around 280 μs reflects the reoxidation of Q_A^- directly by Q_B. The medium phase, Amp_m, with a half-life time of 4.5 ms reflects the binding of plastoquinone to the Q_B site and the subsequent oxidation of Q_A^-. It is therefore determined by the rate of plastoquinone reoxidation by the cytochrome b_6/f complex. The slow phase, with a half-life time of 2 s, is determined by the recombination of Q_A^- with the S_2 states of the water-splitting system. By the exponential analysis of the fluorescence decay kinetics into its different phases it could be shown that the loss of open PS II traps is due to a loss of PS II centres with oxidised Q_B in the binding niche (Table 1) represented by Amp_f. These centres have been transformed into photoinactivated centres unable to reduce Q_A, which converts the excitation energy into heat [35]. The other two components, Amp_m and Amp_s, did not change during illumination.

3.2. Effect of different light intensities on thylakoid protein phosphorylation

To test how increasing photon flux densities up to photoinhibitory conditions affect thylakoid protein phosphorylation, intact chloroplasts were incubated for 10 min in the presence of [^{32}P]orthophosphate and 10 mM $NaHCO_3$. Under these conditions a variety of thylakoid proteins became phosphorylated (Fig. 3A). The main proteins have been identified as the LHC II, the 43 kDa protein of the inner antennae, the D1 and D2 protein and the 9 kDa psbH gene product. A minor but still significant labelling of a polypeptide in the 12 kDa region was also observed.

As is shown by the autoradiogram and its laser densitometrical evaluation (Fig. 3B), steady-state phosphorylation of the LHC II and the proteins belonging to PS II were differentially regulated. LHC II phospho-

Table 1
Effect of raising PFDs on Photosystem II activity in intact spinach chloroplasts determined by fluorescence decay measurements

PFD (μmol m^{-2} s^{-1})	Time (min)	F_0	F_M	Amp_f (rel. units)	Amp_m (rel. units)	Amp_s (rel. units)
Dark control	0	320	845	370	73	82
125	10	317	845	369	78	81
	20	321	899	402	89	87
	30	320	884	393	82	88
500	10	317	822	333	82	90
	20	339	762	261	78	90
	30	359	693	155	94	85
2500	10	320	760	291	71	78
	20	334	698	203	76	85
	30	359	686	163	92	72
3750	10	310	703	254	58	81
	20	327	639	174	48	90
	30	342	583	115	54	72
5000	10	330	688	221	61	76
	20	339	588	123	67	59
	30	344	472	36	61	61

Intact chloroplasts were incubated in assay buffer containing HCO_3^- at 20°C. During incubation chloroplasts were light-treated with photon flux densities of 125, 500, 2500, 3750 and 5000 μmol m^{-2} s^{-1}. From zero time up to 30 min PS II activity was observed every 10 min using fluorescence decay measurements. For the decay measurements each sample was dark-adapted for 5 min.

rylation reached its maximum at intensities of 125 μmol m^{-2} s^{-1}. With increasing light intensities steady-state level of LHC II phosphorylation decreased and at PFDs of 2500 μmol m^{-2} s^{-1} it was only 25% of the phosphorylation level reached at low light. A similar behaviour could also be shown for the 12 kDa protein.

To determine the individual contribution of kinase and phophatase activities on the steady-state level of thylakoid protein phosphorylation, NaF was added to inhibit phosphatase activity and to measure only the kinase activity. The concentrations of NaF have been shown to affect neither electron transport within PS II nor quenching of fluorescence by ΔpH and redox state of the plastoquinone pool. As shown in Fig. 3C steady-state level of LHC II phosphorylation decreased at high light conditions even in the presence of NaF. This shows that indeed LHC II kinase was inhibited by high light intensities. However, in the presence of NaF its steady-state level of phosphorylation was only reduced by 40% at photoinhibitory conditions, whereas the loss in the presence of active phosphatase was about 75%. Obviously, the phosphatase specific for LHC II was activated even more at high light intensities and determined to a major extent the steady-state level of phosphorylation in organello.

In contrast to the LHC II protein family the proteins of PS II were maximally phosphorylated at somewhat higher light intensities of 500 mol m^{-2} s^{-1}. Their phosphorylation level did not decrease with higher light intensities (Fig. 3A). Addition of NaF (Fig. 3C) showed that neither kinase nor phosphatase activity specific for PS II proteins could be influenced by the different light intensities. This is in contrast with the results of Ellich et al. [25] who observed a low light-dependent phosphatase activity specific for PS II proteins in *Spirodela*.

3.3. Initial kinetics of thylakoid protein phosphorylation

In addition to the previous experiments light-induced inhibition of LHC II protein kinase was studied in the first 10 min of illumination to check if it was caused by PS II photoinactivation. Up to this time no significant loss of PS II activity by photoinhibition could be observed even at a PFD of 5000 μmol m^{-2} s^{-1} (Fig. 2). To test kinase activity during this time, intact chloroplasts were incubated with [^{32}P]orthophosphate in the presence of NaF. Starting from zero time of illumination the initial rise of ^{32}P-labelled thylakoid proteins was analysed every minute up to 10 min at three different light intensities (Fig. 4). The increase of ^{32}P-labelled thylakoid proteins started immediately at the beginning of light treatment and could be observed for both PS II and LHC II phosphoproteins. As shown in earlier studies [2,3], the increase of

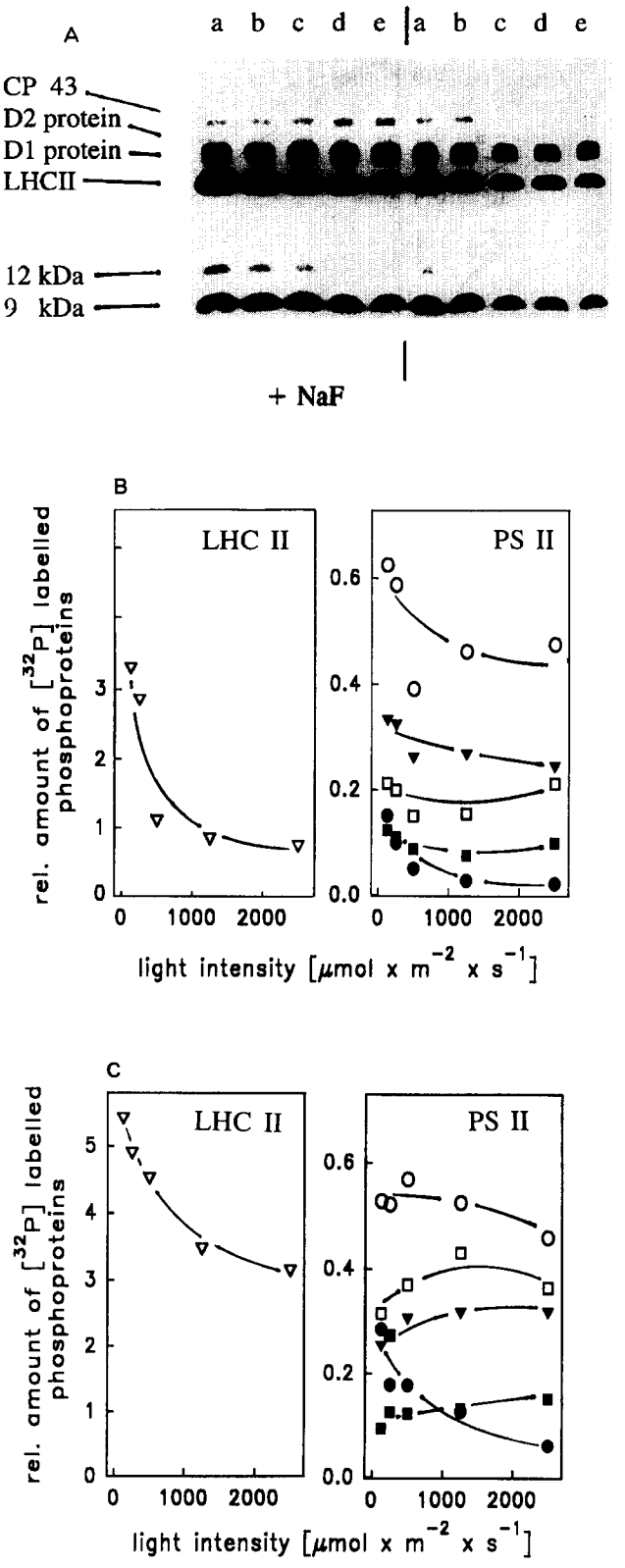

Fig. 3. Effect of different light intensities on thylakoid protein phosphorylation after 10 min in the absence (B) or presence (C) of NaF. The relative amount of ^{32}P-labelled phosphoprotein was determined for LHC II (▽), D1 (▼), D2 (□), CP 43 (■), 12 kDa (●) and 9 kDa (○) by laser densitometric scan of the Western blot autoradiography (A). For this experiment PFDs of 125 (a), 250 (b), 500 (c), 1250 (d) and 2500 (e) μmol m^{-2} s^{-1} were used.

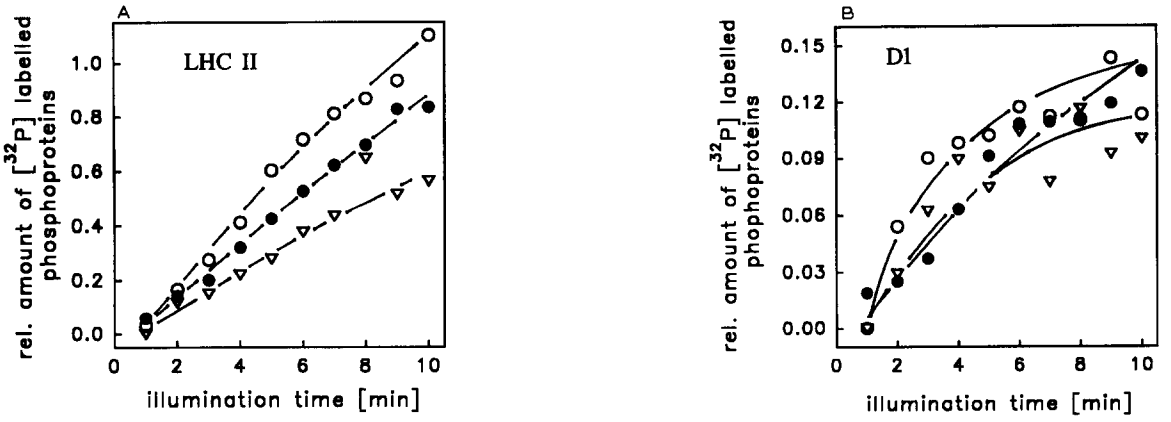

Fig. 4. Effect of different light intensities on the initial phosphorylation of LHC II (A) and the D1 protein (B). The phosphorylation assay was carried out for 10 min in the presence of NaF and [^{32}P]orthophosphate using PFDs of 125 (○), 500 (●) and 2500 (▽) μmol m^{-2} s^{-1}.

^{32}P-labelled LHC II was more than 6-fold faster than labelling of the PS II phosphoproteins. Similar to the previous results, LHC II specific kinase was again inhibited under high light conditions (Fig. 4A). This shows that the activity of the kinase is in some way modulated by light-induced changes. However, its loss of activity cannot be ascribed to a functional inhibition of PS II by photoinactivation, since it occurred already before any functional damage to PS II took place. In contrast to phosphorylation of LHC II, the initial rate of PS II protein phosphorylation was not significantly influenced by the different light treatments (Fig. 4B).

3.4. Recovery of LHC II kinase activity after high light treatment under low light

To test whether LHC II kinase inactivation caused by high light treatment was reversible, recovery of its activity was studied under low light conditions (Fig. 5). First LHC II phosphorylation activity was determined by illuminating intact chloroplasts with PFDs of 200, 1250 and 2500 μmol m^{-2} s^{-1} for 10 min in the presence of NaF (Fig. 5; a, b, c 10 min). Again, illumination at higher PFDs resulted in an inhibition of LHC II phosphorylation. A similar result was obtained when the samples were preilluminated for 30 min at the same PFDs before NaF and [^{32}P]orthophosphate were added for 10 min to determine LHC II phosphorylation. Again, higher light intensities led to a decrease in LHC II phosphorylation (Fig. 5; a, b, c 40 min). However, its level was generally higher. When the same samples illuminated for 30 min with 1250 mol m^{-2} s^{-1} and 2500 mol $^{-2}$ s^{-1}, respectively, were brought back to a PFDs of 200 mol m^{-2} s^{-1} during the 10 min phosphorylation phase, the level of LHC II phosphorylation was higher than in the samples kept at high light. This shows that light-induced inhibition of LHC

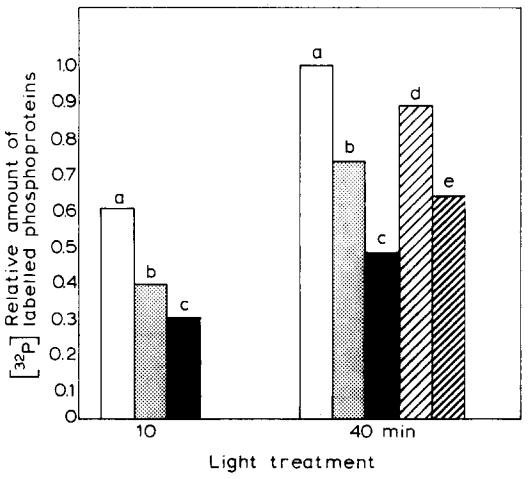

Fig. 5. Recovery of LHC II kinase activity after high light treatment. LHC II kinase activity was observed in the presence of NaF and orthophosphate using PFDs of 200 (a), 1250 (b) and 2500 (c) μmol m^{-2} s^{-1} after 10 min. After a preillumination phase of 30 min, kinase activity was checked again during 10 min incubation under the same light conditions (a, b, c 40 min) in the presence of NaF. Additional samples were treated for 30 min with PFDs of 1250 (d) and 2500 (e) μmol m^{-2} s^{-1}. In the following 10 min phosphorylation assay was carried out in the presence of NaF and [^{32}P]orthophosphate using a low light condition of 200 μmol m^{-2} s^{-1}.

II kinase is reversible and kinase activity can recover on a relatively short time scale.

3.5. Study of thylakoid phosphoprotein phosphatase activity in the dark

In this experiment the rate of dephosphorylation of thylakoid proteins was studied in the dark to determine the protein phosphatase activity specific for LHC II and the phosphoproteins belonging to PS II. After 20 min prelabelling in the presence of [^{32}P]orthophosphate at 500 μmol m^{-2} s^{-1}, dephosphorylation of thylakoid proteins was observed in the dark in intact chloroplasts

(Fig. 6C). At the beginning of the dark treatment phosphorylation assays were divided in samples with or without addition of NaF. In the presence of NaF (Fig. 6A), no dephosphorylation of LHC II was observed. In contrast, a small dephosphorylation of the PS II phosphoproteins could still be detected, showing that the phophatase specific for the PS II proteins was less sensitive towards NaF. In the absence of NaF (Fig. 6B) all thylakoid phosphoproteins became dephosphorylated. However, dephosphorylation of LHC II was much faster than that of the PS II proteins. This is in agreement with the results of Silverstein et al. [21]. Together with the different sensitivity against NaF, this confirms the existence of different phosphatase systems specific for LHC II and PS II proteins.

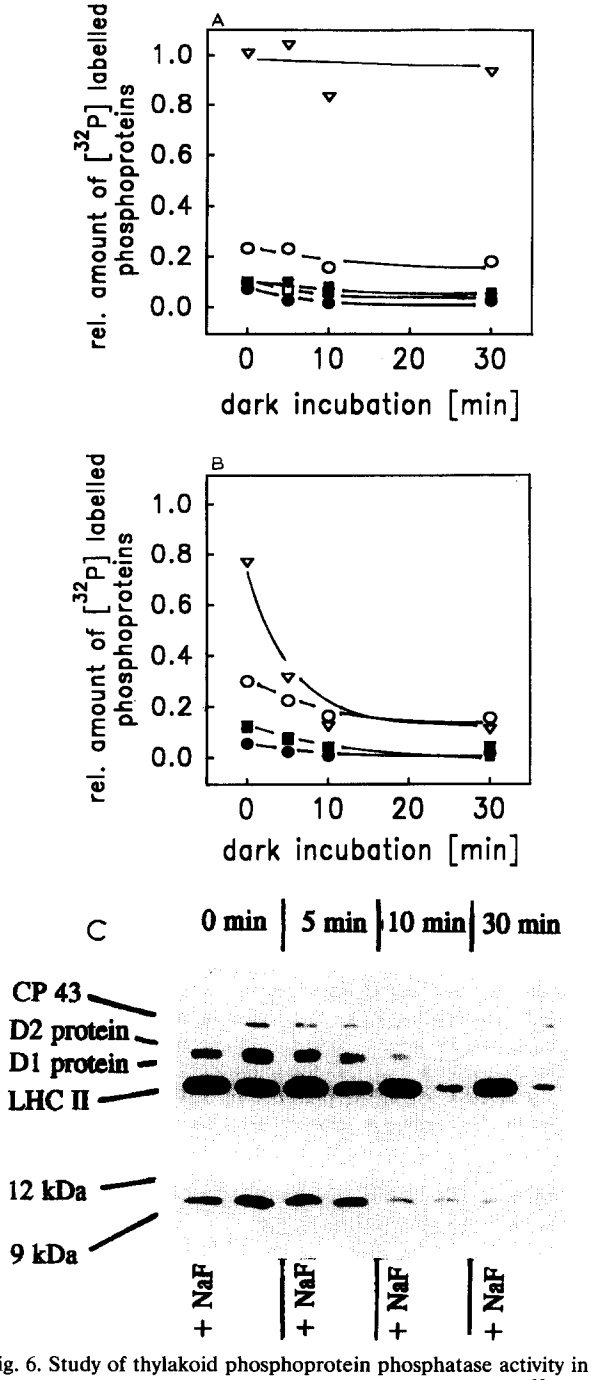

Fig. 6. Study of thylakoid phosphoprotein phosphatase activity in the dark. After 20 min prelabelling in the presence of [^{32}P]orthophosphate at 500 μmol m^{-2} s^{-1} intact chloroplasts were incubated in the dark in the presence (A) or absence (B) of NaF. The relative amount of ^{32}P-labelled phosphoprotein was determined for LHC II (▽), D1 (▼), D2 (□), CP 43 (■), 12 kDa (●) and 9 kDa (○) by laser densitometric scan of the Western blot autoradiography (C). The time scale used for a, b and c represents the duration of treatment in the dark.

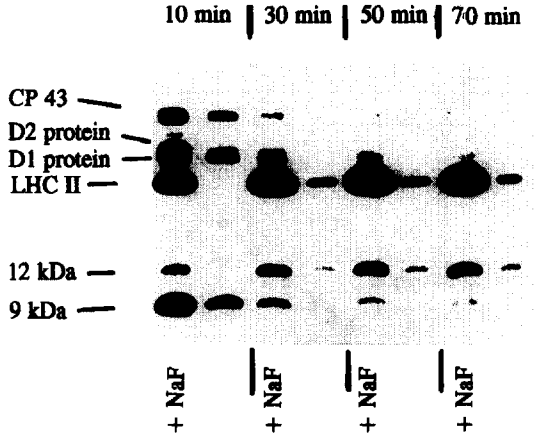

Fig. 7. Possibility of thylakoid protein phosphorylation in intact chloroplasts during increasing times of preillumination (autoradiogram). Intact chloroplasts were illuminated for 0, 20, 40 and 60 min in the absence of NaF at 500 μmol m^{-2} s^{-1}. The samples were illuminated for another 10 min in the presence of [^{32}P]-orthophosphate with or without adding NaF to get total illumination times as mentioned on the top of the figure.

3.6. Phosphate group turnover of thylakoid proteins in intact chloroplasts

To determine the turnover of the phosphate groups during light treatment, intact chloroplasts were preilluminated for 0, 20, 40 and 60 min in the absence of NaF before [^{32}P]orthophosphate was added. The samples were then illuminated for another 10 min in the presence or absence of 10 mM NaF to study both protein kinase activity and steady-state phosphorylation of the thylakoid phosphoproteins. From the autoradiogram (Fig. 7) it can be seen that LHC II phosphorylation was possible at any time during preillumination. This indicates a high and increasing turnover of phosphate groups bound to LHC II. The PS II phosphoproteins became only phosphorylated in dark-adapted chloroplasts which had not been preilluminated. With time of preillumination the level of labelling decreased and after 20 min preillumination no further ^{32}P incorporation into these proteins could be observed. This shows that the phosphate groups once they were esterified to the PS II proteins could not be removed. Obviously, phosphatase activity specific for PS II proteins was very

low in the light. As mentioned before, phosphorylation of the 12 kDa protein showed the same development as found for LHC II. As a result, the phosphorylation level of LHC II is controlled by a high active protein kinase and protein phosphatase. In comparison the PS II protein phosphorylation level during illumination is determined mainly by its protein kinase activity filling up the binding sites.

3.7. Effect of different light intensities on D1 protein synthesis in intact chloroplasts

The low phosphate group turnover found for the PS II proteins was astonishing, since it is known that at least one protein, namely the D1 protein, is characterised by a rapid turnover in light. According to Aro and co-workers [8,18] phosphorylation is thought to regulate the turnover of the D1 protein. To test whether D1 protein turnover is functioning in intact chloroplasts under conditions where phosphorylation had been carried out, they were incubated with [^{14}C]leucine at different PFDs to measure D1 protein synthesis. Other amino acids were not added, since they were also absent during the phosphorylation experiments. Accumulation of the radioactively labelled D1 protein was compared with its actual content determined by Western blotting to get information on the rate of degradation. As shown in Fig. 8, D1 protein synthesis increased up to a saturation point near 500 μmol m^{-2} s^{-1}. Higher light intensities did not result in stimulation of D1 protein synthesis, even when the incubation time was prolonged (data not shown), probably due to limitations by the inner pool size of available amino acids. At a PFD of 500 μmol m^{-2} s^{-1}, PS II became also significantly photoinactivated. The content of D1 protein did not decrease even at light intensities above 500 μmol m^{-2} s^{-1}. This shows that D1 protein synthesis and degradation were strongly coupled. Obviously, at light intensities higher than 500 μmol m^{-2} s^{-1} damage to PS II became so fast that it could not be outbalanced by the limited D1 protein turnover. Thus, accumulation of damaged PS II centres became visible as loss of PS II function. However, although measurable D1 protein turnover existed in the intact chloroplast system, it is not reflected by its phosphate group turnover. As has already been proposed [8,18], we have to assume that the D1 protein, once it is phosphorylated, cannot be degraded by its protease.

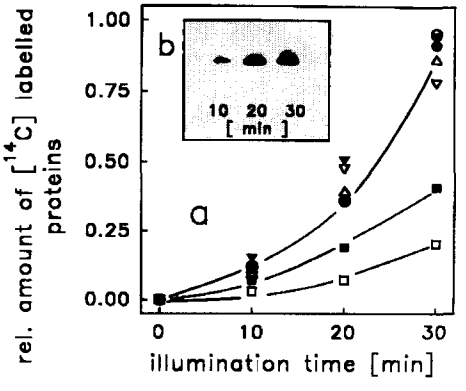

Fig. 8. Effect of different light intensities on D1 protein synthesis in intact chloroplasts. As shown by the graph (a) D1 protein synthesis in intact chloroplasts rises with increasing PFDs from 125 (□), 250 (■), 375 (△), 500 (○), 2500 (●), 3750 (▽) 5000 (▼) μmol m^{-2} s^{-1} up to a saturation point. [^{14}C]Leucine D1 protein labelling pattern of intact chloroplasts (b) is presented for the sample illuminated with a PFD of 500 μmol m^{-2} s^{-1}.

4. Discussion

Previous results [26–28] have indicated that thylakoid protein phosphorylation in vivo is more complexly regulated than can be assumed from the results found in in vitro experiments. Especially the light activiation of phosphatase activity found in Spirodela [26] and the high light dephosphorylation [27,28] of LHC II have only been observed in vivo, but not in isolated thylakoids. The aim of this work was to check how CO_2-fixing chloroplasts control the phosphorylation level of their thylakoid proteins by regulating their kinase and phosphatase activities.

Based on their different sensitivity against inhibitors of the cytochrome b_6/f complex [2] it has long been assumed that LHC II polypeptides and the proteins belonging to PS II are phosphorylated by two different kinase activities. This could be further substantiated by our results. LHC II phosphorylation in the presence of NaF, which inhibits its phosphatase activity, was found to be light sensitive and to decrease with increasing PFD. Also the phosphorylation level of a 12 kDa polypeptide decreased with increasing PFDs. This protein seems to be identical to the one identified by Bhalla and Bennett [37]. The proteins belonging to PS II did not show such a light-induced dephosphorylation. To these group of proteins belong the reaction centre core proteins D1 and D2, the psbH gene product and the 43 kDa polypeptide of the inner antennae. Since the phosphorylation level of PS II stayed stable, it can be excluded that the high-light-induced dephosphorylation of LHC II was due to a decreased phosphate content of the chloroplasts. Thus, we have to assume that kinase activity or substrate availability is different for LHC II and the proteins belonging to PS II.

Light-induced dephosphorylation of LHC II has already been observed under photoinhibitory conditions in green algae and intact leaves [26,27]. It could not be ascribed to an oxidation of the plastoquinone pool due to photoinhibition, since LHC II phosphorylation could

not be reactivated by the addition of duroquinol [26]. Our results from quenching analysis of chlorophyll fluorescence indicated that the plastoquinone pool remained highly reduced even at photoinhibitory conditions. This is in agreement with the measurements of chlorophyll fluorescence relaxation after a single turnover flash which showed that the amount PS II centres with unoccupied Q_B side increased at photoinhibitory conditions. The increase in the reduction state of the plastoquinone pool might be due to cyclic electron transport which has recently been shown to be stimulated at high light conditions [38]. The initial kinetics of LHC II phosphorylation in the presence of NaF showed that high-light- induced LHC II dephosphorylation even precedes photoinactivation of PS II and is rapidly reversible. Therefore, it can be ruled out that a possible oxidation of the plastoquinone pool due to an inhibition of PS II was responsible for LHC II kinase inactivation. Instead, we have to assume that LHC II kinase activity, although principally under redox control of the plastoquinone pool, is modulated by other factors.

Such a factor could be identified as the energetisation of the thylakoid membrane. As indicated by chlorophyll fluorescence analysis, this factor increased with increasing light intensity resulting in the dephosphorylation of LHC II. It also develops fast enough to explain the rapid inhibition of LHC II phosphorylation at high light. Also for other CO_2-fixing systems it has been shown that the demand for ATP and membrane energetisation can alter the phosphorylation level of LHC II [12,14]. The effect of ΔpH on the rate of LHC II phosphorylation is not principally in contradiction with the model of redox control, since it might be just due to changes in LHC II confirmation, and not caused by an inactivation of LHC II kinase itself.

However, inhibition of the LHC II phosphorylation reaction was not the only reason for the decrease of steady-state phosphorylation of LHC II under high light conditions. As shown in this paper, it is also due to an increase of LHC II phosphatase activity. Under high light intensities, the LHC II phosphorylation reaction was inhibited about 40%, whereas the steady-state level of LHC II phosphorylation in the presence of active phosphatase was reduced to another 75%. This shows that in CO_2-fixing systems the increase of phosphatase activity determines mainly the level of LHC II phosphorylation. In vitro, the steady-state level of thylakoid protein phosphorylation depends mainly on the activity of the kinase [1–3]. This is due to the fact that the phosphatases studied in isolated thylakoids, although kinetically different, are not under light or redox control [21,23]. These phosphatases are surely membrane-bound or at least membrane-attached proteins, which are not lost during membrane isolation. The light-stimulated phosphatase activity specific for LHC II might therefore be a stromal protein, which is lost during isolation of thylakoids, and therefore cannot be found in isolated systems.

The function of LHC II dephosphorylation under high light intensities remained still unclear. Phosphorylation disconnects LHC II from the inner antennae core of PS II and increases its mobility in the thylakoid membrane. It may therefore be assumed that the high-light-induced dephosphorylation causes a rebinding of LHC II to the inner antennae core of PS II. However, results from Schuster et al. [26], based on electron microscopic studies, do not support this assumption. They could show that phosphorylation of LHC II results in a reduced PS II particle size, which did not change at photoinhibitory conditions, when LHC II was dephosphorylated again.

The kinase activity responsible for the phosphorylation of the polypeptides belonging to PS II was not significantly regulated by light. All these proteins were maximally labelled at light intensities of 500 μmol m^{-2} s^{-1}. At these light intensities photosynthetic oxygen evolution with CO_2 as acceptor was found to be already light saturated and the reoxidation of Q_A^- was already limited. As indicated by the loss of photochemical quench, Q_A, the plastoquinone pool was in a highly reduced state. This confirms that PS II phosphorylation is under the control of the plastoquinone pool. The energy state of the membrane reflected by q_N, which modulates LHC II kinase activity or substrate availability, obviously had no effect on the PS II protein kinase. This explains why phosphorylation of PS II proteins remained at a high level even at photoinhibitory conditions.

In contrast to LHC II phosphatase, the phosphatase specific for the PS II polypeptides was not active in the light but only in the dark. It also showed a lower sensitivity against NaF, a potent inhibitor of the LHC II phosphatase. Its activity was found to be much lower than the LHC II specific phosphatase which is in general agreement with previous investigations [2,21,39] where half-life times of 6–8 and 30 min were mentioned for the LHC II and PS II phosphoprotein dephosphorylation. Interestingly, no phosphatase activity specific for PS II proteins could be found in the light. Already after 20 min preillumination no more ^{32}P labelling of PS II proteins was found. Thus, due to the absence of a significant phosphate group turnover, PS II phosphoproteins once phosphorylated stayed in this condition for a long time. Observations of Elich et al. [25] in *Spirodela* showed a stimulation of D1 protein dephosphorylation in vivo under low light conditions up to a PFD of 200 μmol m^{-2} s^{-1}. Whether the phosphatase activity that we found in the dark persisted at low light intensities was not tested. However, the results confirm that kinase/phosphatase systems responsible for the LHC II protein family and the PS

II phosphoproteins are different. The different sensitivity to NaF also confirmed the existence of different types of phosphatase in intact chloroplasts.

Phosphorylation of the D1 protein is assumed to play an important role in the regulation of the rapid turnover of the D1 protein [17,18]. Whereas phosphorylation of the D1 protein was first thought to mark it for degradation [17], recent results of the Aro group [18] point towards a stabilisation of D1 by phosphorylation. We could show that the rapid turnover of the D1 protein still occurs in the intact chloroplast system and D1 protein synthesis reached its saturation point at about 500 μmol m^{-2} s^{-1}, the light intensity when photoinhibition started to occur. The constant content of D1 protein all over the light regime showed that D1 protein synthesis was tightly coupled to the D1 protein degradation as has been demonstrated by Adir et al. [40]. Obviously, the D1 protein was already faster degraded before photoinhibition became measurable. This shows that also damage to PS II occurred before the manifestation of photoinhibition. However, this damage could be repaired sufficiently as long as the turnover of the D1 protein could increase. When D1 protein turnover became limited, and the D1 protein was not degraded fast enough [40–42], photoinactivated PS II centres could accumulate. This confirms the significance of the D1 protein turnover as repair cycle of PS II [7,8].

However, D1 protein turnover does not seem to have any effect on the turnover of phosphate groups bound to the D1 protein. Although new D1 protein, which is not phosphorylated [6], was rapidly synthesised throughout, this did not increase the ability of the D1 protein to become phosphorylated. Our results therefore point into the same direction as the results of Aro et al. [8,18] who could show that phosphorylated D1 protein is no substrate for the D1 protein cleavage system. Thus, we suggest that phosphorylation creates a stable pool of PS II centres which is not involved in the PS II repair cycle. The function of this pool is so far unknown. Interestingly, there seems to be a correlation with the degree of phosphorylation and the rate of D1 protein synthesis and both processes reach their maximum at the same light intensity. The same correlation has also been found in intact leaves (data not shown). Thus, phosphorylation and D1 protein synthesis are regulated by the same factor, most likely by the redox state of the plastoquinone pool as has been proposed recently by Allen [43].

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